



Volume  
**110**

*in* **ADVANCES**  
**MEDICINE** *and*  
**BIOLOGY**

*Leon V. Berhardt*  
Editor

NOVA

**ADVANCES IN MEDICINE AND BIOLOGY**

**ADVANCES IN MEDICINE  
AND BIOLOGY**

**VOLUME 110**

No part of this digital document may be reproduced, stored in a retrieval system or transmitted in any form or by any means. The publisher has taken reasonable care in the preparation of this digital document, but makes no expressed or implied warranty of any kind and assumes no responsibility for any errors or omissions. No liability is assumed for incidental or consequential damages in connection with or arising out of information contained herein. This digital document is sold with the clear understanding that the publisher is not engaged in rendering legal, medical or any other professional services.

# **ADVANCES IN MEDICINE AND BIOLOGY**

Additional books in this series can be found on Nova's website  
under the Series tab.

Additional e-books in this series can be found on Nova's website  
under the e-book tab.

**ADVANCES IN MEDICINE AND BIOLOGY**

**ADVANCES IN MEDICINE  
AND BIOLOGY**

**VOLUME 110**

**LEON V. BERHARDT**  
**EDITOR**



Copyright © 2017 by Nova Science Publishers, Inc.

**All rights reserved.** No part of this book may be reproduced, stored in a retrieval system or transmitted in any form or by any means: electronic, electrostatic, magnetic, tape, mechanical photocopying, recording or otherwise without the written permission of the Publisher.

We have partnered with Copyright Clearance Center to make it easy for you to obtain permissions to reuse content from this publication. Simply navigate to this publication's page on Nova's website and locate the "Get Permission" button below the title description. This button is linked directly to the title's permission page on copyright.com. Alternatively, you can visit copyright.com and search by title, ISBN, or ISSN.

For further questions about using the service on copyright.com, please contact:

Copyright Clearance Center

Phone: +1-(978) 750-8400

Fax: +1-(978) 750-4470

E-mail: [info@copyright.com](mailto:info@copyright.com).

### **NOTICE TO THE READER**

The Publisher has taken reasonable care in the preparation of this book, but makes no expressed or implied warranty of any kind and assumes no responsibility for any errors or omissions. No liability is assumed for incidental or consequential damages in connection with or arising out of information contained in this book. The Publisher shall not be liable for any special, consequential, or exemplary damages resulting, in whole or in part, from the readers' use of, or reliance upon, this material. Any parts of this book based on government reports are so indicated and copyright is claimed for those parts to the extent applicable to compilations of such works.

Independent verification should be sought for any data, advice or recommendations contained in this book. In addition, no responsibility is assumed by the publisher for any injury and/or damage to persons or property arising from any methods, products, instructions, ideas or otherwise contained in this publication.

This publication is designed to provide accurate and authoritative information with regard to the subject matter covered herein. It is sold with the clear understanding that the Publisher is not engaged in rendering legal or any other professional services. If legal or any other expert assistance is required, the services of a competent person should be sought. FROM A DECLARATION OF PARTICIPANTS JOINTLY ADOPTED BY A COMMITTEE OF THE AMERICAN BAR ASSOCIATION AND A COMMITTEE OF PUBLISHERS.

Additional color graphics may be available in the e-book version of this book.

### **Library of Congress Cataloging-in-Publication Data**

ISSN: 2157-5398

ISBN: 978-1-53610-485-1

*Published by Nova Science Publishers, Inc. † New York*

# CONTENTS

<b>Preface</b>		<b>vii</b>
<b>Chapter 1</b>	Parkin and Other Proteins of Parkinson's Disease <i>Jolanta Dorszewska, Marta Kowalska, Wiktoria Blaszczyk, Katarzyna Wize and Wojciech Kozubski</i>	<b>1</b>
<b>Chapter 2</b>	Statins and Yeast Polysaccharides in the Treatment of Hyperlipidemia and Liver Steatosis, Role of Autophagy <i>T. P. Johnston, T. A. Korolenko and N. P. Bgatova</i>	<b>31</b>
<b>Chapter 3</b>	Protection Against Mitochondrial and Cell Damage: A New Approach to Treat Severe Hemorrhagic Shock <i>Z. H. Zeng, S. Q. Xu and K. S. Zhao</i>	<b>61</b>
<b>Chapter 4</b>	Telomerase-Targeted Replicative Adenoviruses for Cancer Treatment and Diagnosis <i>Hiroshi Tazawa, Shunsuke Kagawa, Kunitoshi Shigeyasu and Toshiyoshi Fujiwara</i>	<b>95</b>
<b>Chapter 5</b>	Evaluation of the Swelling and Diffusional Behavior of Guar Gum for the Controlled Release of Bioactive Agents <i>John Rojas and Yhors Ciro</i>	<b>115</b>

<b>Chapter 6</b>	The Application of Phytoestrogens in the Cosmetic Industry <i>Alicja Kapuścińska, Anna Olejnik and Izabela Nowak</i>	<b>131</b>
<b>Chapter 7</b>	The Potential Role of Phytoestrogens in Hormonal Replacement Therapy <i>Alicja Kapuścińska, Anna Olejnik and Izabela Nowak</i>	<b>151</b>
<b>Chapter 8</b>	Preventive Measures of Developing Osteonecrosis of the Jaw in Patients Receiving Bisphosphonate Therapy <i>Vladimíra Schwartzová, Peter Kizek and Jozef Minčík</i>	<b>173</b>
<b>Chapter 9</b>	The Oxidative Damage Pathways of Glutamine in Some Clinical Diseases <i>David Calderón Guzmán, Hugo Juárez Olguín, Ernestina Hernández García, Mónica Punzo Soto and Gerardo Barragán Mejía</i>	<b>189</b>
<b>Chapter 10</b>	Glutamine in Sport and Exercise <i>Hércules Rezende Freitas</i>	<b>207</b>
<b>Index</b>		<b>227</b>

## PREFACE

The chapters in this volume present the latest developments in medicine and biology. Chapter One reviews parkin and other proteins of Parkinson's Disease. Chapter Two discusses hyperlipidemia, its impact on the development of cardiovascular disease (CVD), and the use of statins, as well as both yeast polysaccharides and other miscellaneous newer and investigational non-statin agents, to treat hyperlipidemia. Chapter Three summarizes the concept, pathogenesis, variables and treatment of Mitochondrial dysfunction in severe hemorrhagic shock. Chapter Four reviews use of telomerase-targeted replicative adenoviruses for cancer treatment and diagnosis. Chapter Five evaluates the swelling and diffusional behavior of guar gum for the controlled release of bioactive agents. Chapter Six reviews both *in vitro* and *in vivo* studies that were carried out to check the activity of phytoestrogens used in topical products. Chapter Seven presents the potential application of phytoestrogens to alleviate the symptoms of climacteric. Chapter Eight provides preventive measure of developing osteonecrosis of the jaw in patients receiving bisphosphonate therapy. Chapter Nine investigates the possible mechanisms of the protective effect of glutamine on animal models and its use in the clinic. Chapter Ten presents relevant features of the glutamine role in immune pathways, energy metabolism and exercise, highlighting the relationships between glutamine supplementation and performance in adult subjects with different levels of activity.

Chapter 1 - Parkinson's disease (PD) is one of the most common degenerative diseases of the central nervous system (CNS) and affects nearly 2% of the population over the age of 65 and 5% over the age of 85. Moreover, the recent estimates indicate that a number of PD sufferers will increase in time, which is associated with population ageing phenomenon. The pathogenesis of



PD is still not fully understood. It has been found that pathogenesis of this disease may be mediated by proteins, such as alpha-synuclein (ASN), Parkin, leucine-rich repeat kinase-2 (LRRK2) and high temperature requirement protein (HTRA2) and others e.g., protein deglycase (DJ-1) and PTEN-induced putative kinase 1 (PINK1), and TMEM230. Physiologically ASN may modulate catecholamine biosynthesis, by downregulating the expression of tyrosine hydroxylase as well as genes involved in the dopamine (DA) synthesis. Furthermore, structural and functional disorders of the ASN are observed in several neurodegenerative diseases, including PD.

LRRK2 is associated with membrane structures including the outer membrane of mitochondria and synaptic vesicles. It is also involved in vesicular transport and turnover of cellular proteins, as well as the proper functioning of the mitochondria. The overexpression of LRRK2 alone or along with ASN may increase the destructive processes in the PD. Furthermore, dysfunction of mitochondria is also responsible for the changes in the structure of other proteins such as HTRA2 and Parkin.

Parkin may affect the activity of mitochondrial complex, and indirectly may alter the level of oxidative stress. It is believed that Parkin rather protects mtDNA from damage in the oxidative stress conditions and even induces repair mechanism. Moreover, in neurons Parkin may interact with the DA thus contributing to ASN aggregation.

Understanding the interactions of proteins involved in the pathogenesis of PD may contribute to a better knowledge of the causes of disease and early diagnosis and the effective treatment of this disease.

Chapter 2 - Dyslipidemia is one of the major risk factors for the development of cardiovascular disease. Atherosclerosis resulting from hyperlipidemia causes many serious cardiovascular diseases. Statins are generally accepted as a treatment of choice for lowering of low-density lipoprotein (LDL) cholesterol, which reduces coronary heart disease morbidity and mortality. Since statin use can be associated with muscle problems and other adverse symptoms, non-adherence and discontinuation of statin therapy often leads to inadequate control of hyperlipidemia and increased cardiovascular risk. Hence, there is a critical need to identify additional effective hypolipidemic agents that can be used either in combination with statins, or alone, if statins are not tolerated. Thus, newer hypolipidemic agents such as proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors, antisense oligonucleotides, cholesteryl ester transfer protein (CETP) inhibitors, and microsomal triglyceride transfer protein (MTTP) inhibitors, as well as yeast polysaccharides (beta-

glucans and mannans) and compounds derived from plants (phytopharmaceuticals) such as glucomannans, are being investigated.

Macrophages in atherosclerotic lesions are an important class of antigen-presenting cells, initiating an adaptive immune response to oxidized LDL (ox-LDL) antigens. When a statin drug (atorvastatin) was administered to mice prior to inducing acute lipemia with poloxamer 407 (P-407), lipid levels were reduced, but not back to baseline levels observed in controls. However, administration of beta-glucan (a yeast polysaccharide that stimulates macrophages) in combination with atorvastatin to P-407-induced hyperlipidemic mice resulted in normal lipid levels and demonstrated that beta-glucan was not only an effective adjuvant hypolipidemic agent when used with atorvastatin, but also that the combination was more effective than atorvastatin alone.

Recent investigations have revealed that dysfunction of autophagy (lipophagy) is involved in the progression of liver steatosis (in atherosclerosis) and chronic liver diseases such as alcoholic and nonalcoholic steatohepatitis and hepatocellular neoplasia. Hepatic steatosis disturbs autophagic proteolysis through the suppression of both autophagic induction and lysosomal function. Autophagy of lipids in the liver (lipophagy) may possibly have a protective role in the development of liver lipodosis commonly observed in atherosclerosis.

The authors investigated the effect of suppressing the functional activity of macrophages *in vivo* by the administration of gadolinium chloride ( $GdCl_3$ ), which removes a population of large macrophages in the liver and inhibits their rate of endocytosis. Pretreatment of P-407-induced hyperlipidemic mice with  $GdCl_3$  reduced the serum concentration of triglycerides and LDL-cholesterol at 24 hours, which, although speculative, may potentially be due to a hyper-compensatory increase in the endocytosis of lipids by other macrophage pools (e.g., lung and spleen).

In this chapter, the authors will discuss hyperlipidemia, its impact on the development of cardiovascular disease (CVD), and the use of statins, as well as both yeast polysaccharides and other miscellaneous newer and investigational non-statin agents, to treat hyperlipidemia. Autophagy and lipophagy will be discussed in view of lipid accumulation in the liver, as well as the gadolinium-induced model of macrophage depression in experimental biology and medicine. This model allows for the evaluation of the role of liver macrophages in various liver pathologies as it relates to lipid turnover and lipid storage syndromes, which may give rise to new treatment paradigms for liver disease.

Chapter 3 - Severe shock is a life-threatening situation with ineffective of anti-shock treatments. Therefore, the mechanism of severe shock should be

studied in detail to find new approaches to treatment. In severe shock, mitochondrial damage is a common phenomenon among diverse organs, and administration of mitochondrial protectors might represent a new approach for shock therapy. According to the pharmacological effect, drugs targeting mitochondria in severe shock include following aspects: (1) inhibiting mitochondrial permeability transition pore opening; (2) attenuating reactive oxygen species production; (3) modulating inner ion ( $\text{Ca}^{2+}$ ,  $\text{K}^{+}$ ) channels; (4) ameliorating energy substrate metabolism; and (5) activating sirtuin 1/3. Some of these new therapeutic methods have been confirmed in animal studies, which provided a potential clinical application in shock treatment.

Abbreviations: adenine nucleotide translocase, ANT; acridine orange, AO; arteriolar smooth muscle cells, ASMC; adenosine triphosphate, ATP; atractyloside, ATR; cyclic adenosine monophosphate, cAMP; cyclosporine A, CsA; catalase, CAT; cyclophilin D, CyPD; electron-transferring flavin proteins, ETFs; electron transport system, ETS; functional capillaries density, FCD; glutathione peroxidase, GSH-Px; mitochondrial dysfunction, MD; mitochondrial inner membrane, MIM; mitochondrial membrane potential,  $\Delta\Psi\text{m}$ ; mitochondrial outside membrane, MOM; mitochondrial permeability transition pore, mPTP; N-acetylcysteine, NAC; phenyl-tert-butyl nitron, PBN; polydatin, PD; resveratrol, RSV; reactive oxygen species, ROS; superoxide dismutase, SOD; triphenylphosphonium,  $\text{TPP}^{+}$ ; voltage-dependent anion channel, VDAC.

Chapter 4 - Oncolytic virotherapy has recently emerged as a promising antitumor strategy in which tumor-specific cell death is induced by an oncolytic virus. To induce such tumor-specific cell death, tumor specificity for virus replication or infection is needed. Since tumor cells possess unlimited proliferation ability through activation of telomerase, which elongates chromosomal telomeres and prevents the induction of cell cycle arrest and senescence, telomerase activity is a tumor-specific target molecule for the development of an oncolytic virus. The authors recently generated two types of telomerase-specific replication-competent oncolytic adenoviruses, OBP-301 (Telomelysin) and OBP-401 (TelomeScan) for cancer treatment and diagnosis, respectively. When OBP-301 infects tumor cells, it induces cell lysis. On the other hand, when OBP-401 infects tumor cells, it induces the expression of green fluorescence protein (GFP). This chapter focuses on the recent advances in evaluating the therapeutic potential of OBP-301 in oncolytic virotherapy and the diagnostic potential of OBP-401 for tumor cell detection systems in preclinical and clinical settings. The potential application of OBP-301-based oncolytic virotherapy is discussed in combination therapy with conventional

chemotherapy and radiotherapy. The fluorescence-guided tumor detection system using OBP-401 is discussed in terms of the analysis of circulating tumor cells (CTC). A better understanding of the precise molecular mechanism of the OBP-301-mediated tumor suppression system would provide novel insights for the improvement of OBP-301-based oncolytic virotherapy. Furthermore, an OBP-401-based system for capture of GFP-positive CTC in blood samples would improve the assessment of patients with premetastatic advanced cancers.

Chapter 5 - Guar gum is a hydrophilic polymer that has gained attention for the fabrication of matrices for controlled solute release due to its gelling nature and ability to entrap the solute within the gel. It is highly soluble in water, stable over a wide range of temperature, acidic, alkaline and enzymatic conditions. Matrix systems are made of polymeric materials that are swellable in the presence of biological fluids. The powdered solute was distributed uniformly in a matrix of guar gum and directly compressed to form a tablet using a single punch tablet press equipped with a flat-faced tooling (13 mm diameter). A guar gum matrix tablet is cost effective and have broad FDA acceptance.

The swelling characteristics of guar gum matrix tablets were studied using three solutes having different solubilities. Thus, methylene blue, caffeine and salicylic acid were used as soluble, highly soluble and acidic compounds. Swelling was assessed by measuring the axial and radial expansion of matrix tablets following exposure to distilled water, acidic and brine media. The mechanisms of solute release and matrix swelling rate were calculated from the dissolution and swelling experiments, respectively. Matrix swelling was related to the intake of a large amount of water and formation of a viscoelastic mass. A rapid absorption of water took place through permeation and capillary action. Solute release kinetics included mainly relaxation rather than diffusion transport. Solute release was also influenced by the presence of ions and pH of the media. The mechanism of solute release from this polymeric matrix mostly conforms to non-Fickian (anomalous) transport. Thus, swelling played an important role to obtain complete solute release within 24 h. Further, the ionic strength of the liquid had a strong effect on the sorption properties of the matrix.

Solute release from guar gum matrices was preceded mainly by a combination of swelling and diffusion mechanisms depending on the chemical nature of the solute employed. Kinetics of solute release from these matrices depended mainly on the synchronization of polymer hydration at the moving rubbery/glassy front within the matrix and the rate of solute diffusion.

Chapter 6 - Phytoestrogens, known also as “youth hormones”, are interesting cosmetic innovative ingredients. These compounds are structural analogues of 17- $\beta$ -estradiol, a steroid and estrogen sex hormone. The most

important and well-known class of phytoestrogens are isoflavones. Other classes of phytoestrogens such as lignans, stilbenes and coumestans have also been identified. It is expected that phytoestrogens exhibit anti-aging activity and may be applied in cosmetic products in glycoside forms of isoflavones. Much effort has been made to investigate the potential use of phytoestrogens as cosmetic active ingredients. Topical application of these compounds may be helpful in protecting the skin from free radicals and loss of firmness caused by decreasing collagenase activity. According to literature, the most significant isoflavone, genistein, has an inhibitory activity against protein called tyrosine kinase. Kinases are involved in transduction of signal activated by free radicals and inflammatory cytokines, that leads to the expression of enzymes responsible for the degradation of collagen and elastin, being a key factor causing a decrease in elasticity of skin and skin aging, therefore, the aim of this chapter is to present the application of different phytoestrogens in cosmetic formulations. The information regarding the effectiveness of phytoestrogens as cosmetic active ingredient is included. The goal of this chapter is to review both *in vitro* and *in vivo* studies that were carried out to check the activity of phytoestrogens used in topical products.

Chapter 7 - Menopause is the time in most women's lives when menstrual periods stop permanently. This process typically occurs between 45 and 55 years of age and entails accelerating aging of body. It is caused by a decrease in estrogens production by the ovaries. In order to alleviate the signs of menopause, oral supplementation of estrogens may be recommended. However, according to recent results, not all women can be treated with medicines containing estrogens. For those who cannot, an oral supplementation with phytoestrogens may be considered. The most important and well-known class of phytoestrogens are isoflavones. Phytoestrogens are natural plant hormones that are structural analogues to 17- $\beta$ -estradiol. The mechanism of action of phytoestrogens, especially isoflavones, is based on structural similarity to 17- $\beta$ -estradiol. In this article the authors discuss the potential application of phytoestrogens as an alternative to hormone replacement therapy. The evidence of isoflavones effectiveness in the treatment for menopausal symptoms is reviewed.

Chapter 8 - Osteonecrosis of the jaw is a rare, but very severe complication in patients receiving bisphosphonate therapy. This complication significantly restricts food intake and reduces their quality of life. The negative influence of bisphosphonates on jaw bones is still not precisely known and is the subject of research.

The authors present the increasing number of negative effects of bisphosphonates when used orally or parenterally. Treatment is protracted and very complicated. The authors present clinical symptoms and treatment options based on their own results, while highlighting the most interesting cases from the treatment group.

In the final part of the article, the authors emphasize the significance of preventive measures. In accordance with other studies, the best way to prevent osteonecrosis of the jaw is to observe set precautions (especially before the bisphosphonate treatment initiates).

Chapter 9 - Glutamine is an on-essential amino acid consumed for protein synthesis. It is a substrate for the synthesis of glutathione, the most abundant intracellular thiol and antioxidant. It plays an important role in protecting cells from oxidative stress or apoptosis induced by different diseases. In the present review, the authors investigated the possible mechanisms of the protective effect of glutamine on animal models and its use in the clinic.

Chapter 10 - L-glutamine, a nonessential neutral amino acid, is a key element in several metabolic pathways. Recent investigations have been focused mainly on energy metabolism, cellular proliferation and immune functions of glutamine. Athletes and exercise practitioners regularly supplement this amino acid as a resource to optimize physical performance and prevent immune impairment. Also, studies indicate that acute doses of glutamine, reaching up to 0.65 g/Kg/day (45.5 g for a 70 Kg individual), are well tolerated and do not result in abnormal ammonia levels. This chapter presents relevant features of the glutamine role in immune pathways, energy metabolism and exercise, highlighting the relationships between glutamine supplementation and performance in adult subjects with different levels of activity. In sport, data suggest that glutamine supplementation may increase tolerance to intermittent exercise, lower levels of fatigue, enhance physical and performance measurements, optimize recovery from muscle damage and prevent suppression of neutrophil function, however, these effects are highly dependent on the characteristics of exercise protocols, experimental subjects and doses provided. Data indicating higher levels of nasal and intestine IgA, prevention of hyperammonemia, protection against lymphocyte apoptosis, increase in exercise-induced plasma IL-6 levels and inhibition of cytokines production (NF- $\kappa$ B pathway) through glutamine supplementation suggest a mechanism of immune communication, and novel research strongly points to the importance of immunological exchange in the modulation of performance. Glutamine, however, may be deleterious to the nervous system under disease conditions, such as in hyperammonemia associated with acute liver failure, where excess

glutamine leads to cerebral edema. Therefore, supplementation or pharmacological interventions should be weighted according to one's health status and level of activity.

*Chapter 1*

## **PARKIN AND OTHER PROTEINS OF PARKINSON'S DISEASE**

***Jolanta Dorszewska<sup>1,\*</sup>, Marta Kowalska<sup>1</sup>,  
Wiktoria Blaszcak<sup>1</sup>, Katarzyna Wize<sup>1</sup>  
and Wojciech Kozubski<sup>2</sup>***

<sup>1</sup>Laboratory of Neurobiology, Department of Neurology,

<sup>2</sup>Chair and Department of Neurology,  
Poznan University of Medical Sciences,  
Poznan, Poland

### **ABSTRACT**

Parkinson's disease (PD) is one of the most common degenerative diseases of the central nervous system (CNS) and affects nearly 2% of the population over the age of 65 and 5% over the age of 85. Moreover, the recent estimates indicate that a number of PD sufferers will increase in time, which is associated with population ageing phenomenon. The pathogenesis of PD is still not fully understood. It has been found that pathogenesis of this disease may be mediated by proteins, such as alpha-synuclein (ASN), Parkin, leucine-rich repeat kinase-2 (LRRK2) and high

---

\* Corresponding author: Jolanta Dorszewska, MDs, PhD, Laboratory of Neurobiology, Department of Neurology, Poznan University of Medical Sciences, 49 Przybyszewskiego St., 60-355 Poznan, Poland, Phone: + 48 61 86 91 439, Fax: + 48 61 86 91 697, Email: dorszewska.j@yahoo.com.



temperature requirement protein (HTRA2) and others e.g., protein deglycase (DJ-1) and PTEN-induced putative kinase 1 (PINK1), and TMEM230. Physiologically ASN may modulate catecholamine biosynthesis, by downregulating the expression of tyrosine hydroxylase as well as genes involved in the dopamine (DA) synthesis. Furthermore, structural and functional disorders of the ASN are observed in several neurodegenerative diseases, including PD.

LRRK2 is associated with membrane structures including the outer membrane of mitochondria and synaptic vesicles. It is also involved in vesicular transport and turnover of cellular proteins, as well as the proper functioning of the mitochondria. The overexpression of LRRK2 alone or along with ASN may increase the destructive processes in the PD. Furthermore, dysfunction of mitochondria is also responsible for the changes in the structure of other proteins such as HTRA2 and Parkin.

Parkin may affect the activity of mitochondrial complex, and indirectly may alter the level of oxidative stress. It is believed that Parkin rather protects mtDNA from damage in the oxidative stress conditions and even induces repair mechanism. Moreover, in neurons Parkin may interact with the DA thus contributing to ASN aggregation.

Understanding the interactions of proteins involved in the pathogenesis of PD may contribute to a better knowledge of the causes of disease and early diagnosis and the effective treatment of this disease.

**Keywords:** Parkin, ASN, LRRK2, HTRA2, DJ-1, PINK1, TMEM230, Parkinson's disease

## INTRODUCTION

Parkinson's disease (PD) is the second most common disease of the central nervous system (CNS). It is manifested by impairment of motor (bradykinesia, tremor, postural instability, rigidity), and nonmotor functions. PD is divided, depending on the time of onset of disease, into: late onset PD (LOPD) occurring at the age above 40 years of age and early-onset PD (EOPD) less than 40 years of age. The main factor in PD pathology is a progressive degeneration and subsequent loss of dopaminergic neurons of the nigrostriatal and hypothalamic pathway, along with noradrenergic, serotonergic, and cholinergic neurons, however in varying degrees [1-3].

Pathogenesis of PD depends on both environmental and genetic factors. Currently, there are many hypotheses concerning PD pathomechanism. One of them regards accumulation of pathological proteins as main factor leading to metabolic changes in CNS and development of degenerative process. In familial

PD (FPD) pathological proteins are the result of mutations in encoding genes, while in sporadic PD (SPD) they occur due to posttranslational modifications [4]. According to another theory, dysfunction of the ubiquitin-proteasome system (UPS) leads to the formation of Lewy bodies (LB) and subsequently to neuronal damage and death [5].

LB are found in various parts of the brain of patients with PD: substantia nigra, olfactory departure, neocortex, brain stem and spinal cord or peripheral autonomic system [6, 7]. LB in PD patients were observed also in the cholinergic neurons of the basal ganglia Meynerta, amygdala and hippocampus, noradrenergic neurons of the locus coeruleus and adrenergic, serotonergic neurons, dorsal motor nuclei of the vagus nerve. PD with or without dementia, also revealed the presence of LB in the peripheral nervous system, and in 70% of patients also in the neurons of the skin [8]. In patients without motor symptoms, LB were present in neurons of the autonomic nervous system of the heart, enteric nervous system and the sympathetic cervical ganglia [9]. Moreover, presence of LB in the peripheral autonomic system may, for many years ahead, precede the development of motor disorders in PD.

Since the early 60s of the last century L-dopa remains the most common agent in PD treatment [10]. L-dopa administration induces adverse effects, including dyskinesia, ailments of the digestive system, and blood pressure decrease [11, 12]. Main problem concerning L-dopa therapy is low availability of this amino acid to the CNS. Bouhaddi et al. [13] showed that when L-dopa is administered orally in therapeutic doses, almost all of it - 95% is metabolized peripherally, and only 5% passes through blood-brain barrier, and further can be converted to the DA. The peripheral L-dopa degradation may lead to impaired carboxylate circulation (hyperhomocysteinemia), thus augmenting the immune system response and oxidative stress through different mechanisms [14].

## **THE ROLE OF LEWY BODIES IN PATHOGENESIS OF PARKINSON'S DISEASE**

According to analysis of the LB structure, they are composed of numerous, ubiquitinated proteins, such as cytoskeletal elements, parts of the ubiquitin-proteasome complex, the ubiquitin (Ub) monomers, alpha-synuclein (ASN), Parkin and tau protein (Table 1) [5].

Parkin is an E3 ubiquitin ligase, which is a part of the UPS that mediates targeting of proteins for degradation. Parkin is consisted of unique parkin domain (UPD), ubiquitin-like domain (UBL) at the N terminus, two ring-finger motifs (RING1 and RING2) and Cys-rich domain in-between RING (IBN) at the C terminus. The RING box is built of UBL and RING1-IBN-RING2 [15]. Parkin is located in the cytosol in catalytically inactive form. Activation occurs due to phosphorylation by the protein kinase PINK1 or by phosphoubiquitin, which competes with the inhibitory UBL domain and stabilizes the active form of Parkin [16].

Parkin regulates actin filaments dynamics, autophagy of damaged mitochondria and biosynthesis of new ones [17, 18]. Moreover, Parkin is also one of the mitochondria protective factors. Its overexpression leads to increased expression of complex I subunits and reduced accumulation of reactive oxygen species (ROS) [19]. Parkin can interact with many other proteins e.g., ASN, protein deglycase (DJ-1) and PTEN-induced putative kinase 1 (PINK1).

The posttranslational modifications (PTMs) of Parkin regulate its localization, activity, solubility and substrate selection. Phosphorylation or S-nitrosylation due to stress condition leads to inactivation of Parkin and accumulation of Parkin substrates (like ASN). The consequence is death of dopaminergic neurons. This situation is a hallmark of SPD [20].

Parkin is encoded by *PRKN* (PARK2) gene consisting of 12 exons. Mutations in *PRKN* gene were identified as a cause of autosomal recessive juvenile parkinsonism [21]. They are frequent and are responsible for approximately half of FPD cases and 15% of SPD with EOPD. The mean age of PD onset in patients with *PRKN* mutations is 30 years old, although the cases with late-onset (LOPD) up to 70 years old were also described [22, 23].

More than 100 mutations have been identified in *PRKN* gene. They include deletions, insertions, point mutations and large rearrangements. Almost half of them are missense/nonsense mutations. Although mutations were identified in all exons and 3'UTR region, the most common are in exons 2, 4, 7, 8, 10 and 11 [24, 25]. It still remains unclear how mutations in *PRKN* contribute to nigral cell death.

The mutations in *PRKN* can alter the proper function of Parkin by impaired phosphoubiquitin-induced activation, autoinhibition, loss of activity, decrease in protein stability, misfolding, insolubility, and conformation or charge change. Some of them lead to aggregation of Parkin, while others seem to have no significant effect [26, 27].

Missense mutations in the RING box (e.g., T240R or T415N) lead to almost complete loss of E2-binding activity and may cause loss of ubiquitin-protein

ligase activity and impair Parkin degradation by defects in self-ubiquitination [28]. None of those pathological genetic changes are located outside the ring-finger domains, what indicates that these motifs are crucial to maintaining the physiological function of Parkin.

Interestingly, Kay et al. [29, 30] have shown that heterozygous point mutations in *PRKN* gene are as frequent in PD patients as in control group. However, none of 1,686 control individuals was homozygous or had biallelic mutations (point mutations/deletions/multiplications/copy number variations). The impact of single heterozygous mutations on PD still needs to be researched.

The response to L-dopa in patients carrying *PRKN* mutations is usually good but leads to L-dopa-induced dyskinesia during treatment. Moreover, treated patients are prone to develop dystonia, hyperreflexia and symmetric signs at onset, without dementia. The pathological process is limited to the brainstem. These are not specific features of the disease that differ individuals with *PRKN* mutations from those with others EOPD genes mutations [23, 31].

The study of Vergara et al. [17] has proved that changes in Parkin can affect the expression of cytoskeletal proteins which leads to altered structure and plasticity of fibroblasts. The neuroimaging studies indicated that atrophy in the caudate nucleus may be specific for Parkin changes. The decrease in the volumes of the basal ganglia structures was observed in EOPD patients with *PRKN* mutations, but not in EOPD without mutations in *PRKN* [32].

Another protein involved in the structure of LB, is Ub. This relatively small protein is present in all eukaryotic cells. It is produced from ubiquitin precursors protein encoded by few genes: *UBB*, *UBC*, *UBA52* and *RPS27A*. The covalent addition of Ub to a lysine of a substrate protein is called ubiquitination. Ubiquitination needs three main enzymes to occur: E1, E2 and mentioned E3 ubiquitin-protein ligase, such as Parkin. E1 enzyme activates Ub (by phosphorylation), which is next transferred to E2 ubiquitin-conjugating enzyme. E2 with E3 ubiquitinate substrate proteins [33].

Monoubiquitination occurs, when only single Ub is attached while during polyubiquitination, Ub molecule is added to a few lysine residues on the target protein. Both processes are carried out by Parkin [34]. Both processes however, serve different purposes, e.g., monoubiquitination of Lys-48 is essential for signaling and receptor trafficking, while polyubiquitination leads to proteasomal degradation. [33]. UPS has many other functions, such as response to oxidative stress and DNA damage, regulation of apoptosis or progression of the cell cycle [35].

UPS impairment plays important role in neurodegeneration. Failure of UPS may be a consequence of downregulation and/or modification of proteasomal subunits, increased level of aggregates or reduction in ATP levels subsequent to oxidative damage [36]. Inhibition of the UPS leads to reduced synaptic plasticity and loss of synaptic transmission [35]. The most frequent PTMs of Ub is phosphorylation. Different isoforms of Ub were found in PD lesions.

ASN, other component of LB is a presynaptic terminal protein that in physiological state is non-folded, highly soluble and thermostable. It seems that ASN modulates synaptic plasticity, synthesis and release of neurotransmitters and regulation of follicular transport. ASN is believed to have anti-apoptotic properties. ASN regulates DA homeostasis: it is involved in differentiation and survival of progenitor cells of dopaminergic neurons, inhibits DA transporter and alters the DA uptake rates. On the other hand, oxidized DA promotes ASN aggregation [37, 38]. ASN is degraded mostly by UPS and alternatively by autophagy or cytoplasmic proteases. Parkin due to difference in ubiquitination mechanism (Lys-48 or Lys-63) decides which way of degradation will be chosen [39]. Overexpression of Parkin can protect cells against ASN-induced toxicity. However, studies performed on ASN transgenic model have shown that lack of Parkin does not enhance ASN-induced toxicity [20].

ASN and tau protein can interact with each other and induce their fibrillation [38]. Abnormal accumulation of insoluble ASN and its incorrect proteolytic processing is known to be one of the causes of neurodegeneration. The ASN is a major component of LB [5, 40]. Alterations of ASN structure and PTMs enhance the ASN aggregation. It is known that tyrosine nitration in ASN is a result of oxidative stress and it affects ASN solubility and therefore may contribute to oligomere stabilization. The nitration in ASN acts like A30P mutation of *SNCA* (PARK1, PARK4) gene, described below. Tyrosine phosphorylation of ASN leads to its aggregation and toxicity, also DA modification of ASN stabilizes oligomer formation [41, 42]. Following PTMs: phosphorylated, ubiquitinated, cut at C terminus and oxidized ASN was observed in alpha-synucleinopathic brains and within LB. Interestingly, more than 90% of total ASN in LB is phosphorylated in position Ser-129, while in physiological condition pS129 represents only 4% of ASN. Thus, it was suggested that modified ASN may be better biomarker of PD than total ASN levels [41]. The review of Barrett and Greenamyre [42] underlines that exact role of pS129 remains unclear. According to *in vitro* studies it enhances LB formation and toxicity, while study on *in vivo* rat model suggests that pS129 has a protective role.

The mutations in *SNCA* gene, encoding ASN, are very rare, but have underlined the role of ASN in LB formation. The first mutations, A53T and A30P *SNCA*, were identified in late 90's in European families. [43, 44]. Third mutation, E46K, was found in 2004 in Spanish family [45]. The clinical phenotype of PD patients with *SNCA* mutations is EOPD with LB, autonomic dysfunction, behavioral changes, fast progression and dementia. Those symptoms may be explained by widespread deposition of ASN in the brainstem and entire cerebrum, both in neurons and glial cells. These mutations are linked to increased synaptic vesicle permeability and elevated levels of DA in cytosol. Both, A30P and E46K have not been observed in other families worldwide, whereas A53T has been found in Caucasian and Asian kindred [42-46]. The A53T mutation leads to self-aggregation of ASN due to disruption in its structure. What is more, this mutation is associated with greater *in vivo* toxicity than other ASN variants, but it does not cause mitochondrial complex I deficiency [40, 43].

Triplications and duplications of *SNCA* are more common than point mutation described above. Only duplications of *SNCA* are characterized by slow progression with lack of dementia [46]. Contrary, there were also patients with duplication that manifested very quick progression of PD and no response to L-dopa treatment. The reason of these differences may be variable penetration of *SNCA* duplication [37]. Triplication is associated with a twofold higher ASN level [47].

Not only mutations in *SNCA* gene, but also single nucleotide polymorphisms (SNP) are associated with PD. It was described that SNPs (rs2736990, rs356165 and rs356219) are a risk factor for PD development. One of the polymorphisms - rs356219, correlates with increased concentration of plasma ASN in PD patients. Moreover, alterations in polymorphic promoter region of *SNCA* (NACP-Rep1) increase risk of PD. [24, 47]. There are five alleles of NACP-Rep1 (-1, 0, +1, +2, +3). The +1 allele is the most common genotype in European population and is known to reduce the risk of PD. Moreover, +1/+1 genotype is associated with lower ASN level detected in blood [37, 48].

Leucine-rich repeat kinase 2 (LRRK2) is multiple-domain enzyme localized in cytosol and on mitochondrial outer membrane. Cytosolic form of LRRK2 is monomeric and almost inactive, while active form is dimeric [49]. Its exact function remains unknown. Possibly, it may be involved in cell signaling and synaptic vesicle endocytosis. LRRK2 might play a role in immune response as it does in phagocytosis. Significant overexpression of LRRK2 was detected in immune cells [50].

**Table 1. Genes implicated in monogenic Parkinson's disease. Modified on the basis of Bonifati, 2014 [23] and Puschmann, 2013 [46]**

Gene (locus)	Location	Mutations and/or polymorphisms	Inheritance	Pathological features	Clinical phenotype
<i>PRKN</i> (PARK2)	6q25.2-q27	>100	Recessive	No LB in most cases, low risk for cognitive symptoms, cell loss in brain stem, usually no cortical pathology.	EOPD, slow course
<i>PINK1</i> (PARK6)	1p35-p36	W437X, Q456X, G309D, V170G	Recessive	LB? cognitive and psychiatric symptoms describe, cell loss in brain stem/SN	EOPD, slow course
<i>DJ-1</i> (PARK7)	1p36	M26I, L166P, A104T, D149A	Recessive	Low risk for cognitive symptoms, another features are unknown	EOPD, slow course
<i>HTRA2</i> (PARK13)	2p12	G399S, A141S	Dominant	-	-
<i>SNCA</i> (PARK1, PARK4)	4q21-q23	A53T, A30P, E46K, triplications and duplications	Dominant	LB, cognitive decline, behavioral and autonomic symptoms	EOPD, aggressive course
<i>LRRK2</i> (PARK8)	12p11q13.1	Y1699C, R1441C, R1441G, R1441H, I2020T, N1437H, G2019S	Dominant	LB, highly variable pathology	LOPD

LB - Lewy bodies, EOPD - early-onset Parkinson's disease, LOPD – late-onset Parkinson's disease

Mutations in *LRRK2* (PARK8) gene were identified in families from a few countries from Europe, USA and Japan, and they are thought to be a cause of

10% autosomal dominant FPD. Most of them are rare (Y1699C, R1441C, R1441H, I2020T, N1437H), while two are relatively common in some populations (R1441G, G2019S). The R1441G was found in 8% of FPD individuals from the Basque population. The G2019S was observed in 41% of SPD and 37% of FPD individuals from North African Arab population and 18.3% of Ashkenazi Jewish PD patients [23, 46]. The G2019S is also the most common mutation of *LRRK2* among Caucasians PD patients, while it is extremely rare in controls [51, 52]. Classical LBs are observed in most of *LRRK2* mutation carriers and the age of onset is late. The clinical features of PD in patients with G2019S mutation are more benign. This variant is associated with increased phosphotransferase activity of protein [52]. According to independent studies the mean age of onset was between 53-56 years. All patients carrying G2019S mutation suffered from bradykinesia, rigidity and responded well L-dopa treatment. Postural instability, dyskinesia and resting tremor were observed in most of them [51, 53]. Penetrance of this mutation increases with age: from 17% at age 50 to 85% at age 70 years [51].

One hypothesis indicate that G2019S *LRRK2* mutation may improve the resistance of intracellular pathogens and lead to impaired immune system response [50]. As *LRRK2* activity is increased in G2019S mutation, it is proposed that inhibiting enzyme activity may be a promising target in new PD treatment.

## **OXIDATIVE STRESS AND PATHOGENESIS OF PARKINSON'S DISEASE**

Oxidative stress is regarded as one of main causes of neurodegenerative diseases including PD. It is defined as disturbance in the balance between prooxidant and antioxidant homeostasis, which also takes part in generation of ROS, which are known to be potentially toxic for neuronal cells [54]. Mutations in several PD-associated genes, like *LRRK2*, *HTRA2*, *PINK1*, *DJ-1* and *PRKN* are connected with impaired function of the products of these genes and mitochondrial dysfunctions, cytotoxicity and apoptosis.

Parkin is a part of multiprotein E3 ubiquitin ligase complex and is involved in protein degradation mediated by proteasome [55]. Moreover, its overexpression causes increase in mitochondrial membrane potential, the overexpression of mitochondrial complex I subunits and reduced accumulation of ROS in transfected cells' mitochondria [19]. Parkin also stimulates



mitochondrial fusion of mitophagy as a response of moderating stress conditions [56]. Currently, it is believed that gene polymorphisms of *PRKN* gene, such as G601A, C924T, G1281A, C1305T, can play an important role in the pathogenesis of PD (Table 1). Those mutations are associated with lower enzymatic activity, when arginine is substituted by tryptophan on the domain R1, what is induced by C924T mutation and the accumulation non-ubiquitinated proteins, which cannot take the form of LB [57].

Familial associated mutations of *PRKN* gene cause losing activity of the E3 ubiquitin ligase and suggest autosomal recessive FPD [58]. Mean age of onset is 32 years for the Caucasian population [23]. These mutations occur in up to 50% of those patients younger than 25 years old and cause EOPD [59]. The report by Farrer et al. [57] suggests that compound heterozygous *PRKN* mutations and loss of Parkin expression may lead to early-onset parkinsonism with LB pathology, however, homozygous mutation may confer increased susceptibility to typical PD. Patients with *PRKN* mutation have good result to L-dopa treatment and slow progression of disease [60].

The other protein with impact on oxidative stress is PINK1, encoded by the *PINK1* gene. PINK1 protects against mitochondrial dysfunction during cellular stress by being a damage sensor. It is also engaged in degradation of impaired mitochondria by activation this organelles' autophagy (mitophagy) by Parkin and regulation of Parkin localization [61]. Morais et al. [62] show that *PINK1* modifications, may result in structural and functional changes in mitochondria, elevated ROS generation and enhanced sensitivity to oxidative stress. There are many mutations in *PINK1* including large deletions, frameshift mutations, nonsense (W437X and Q456X) or missense (G309D and V170G) mutations, which cause loss of protein function.

Bonifati et al. [63] identified homozygous pathogenic mutations in the *PINK1* gene in four of ninety Italian sporadic EOPD patients. The disease is manifested by slow progression, excellent response to L-dopa and patients benefit from sleep. Heterozygous mutations have been observed in another four SPD patients. Their phenotype was similar, but onset of the disease occurred later. The Albanese et al. [64] described clinical phenotype of a patient with *PINK1* genetic variant, who manifested typical PD symptoms (right-side prevalence and good response to L-dopa), but lacked dystonia, L-dopa-induced dyskinesias, and hyperreflexia, which are characteristic for EOPD. According to authors this phenotype was not distinguishable from idiopathic form of PD. Toft et al. [65] claims that increased risk of PD development is associated with heterozygous mutations of *PINK1*. The neuropathology report shows that mutated *PINK1* induces LB formation, which are crucial in PD pathobiology.

DJ-1 is a mitochondrial peroxidase. Studies have shown that this protein is of multiple functions and may contribute to prevention of PD. DJ-1 can protect cells against oxidative stress and subsequent death by being oxidative stress sensor and redox-sensitive chaperone and protease [66, 67]. It holds protective capacity against metal induced toxicity through interactions with PINK1, hydrogen peroxide elimination, regulation of inflammatory response and binding of copper and mercury. [68]. DJ-1 also takes part in autophagy regulation during oxidative stress. Therefore, DJ-1 maintains mitochondria functional [69].

The mutations in *DJ-1* gene are associated with autosomal recessive EOPD. It is supposed that homozygous or compound heterozygous mutations lead to loss of DJ-1 function. Some of altered proteins, as M26I and L166P, are unstable, susceptible to degradation by proteasome system, and present reduced ability to eliminate exogenous hydrogen peroxide, which correlates with increased sensitivity to oxydative stress [70]. Mutations A104T and D149A *DJ-1* gene are connected with loss of protective properties against metal cytotoxicity, but C106A maintains those abilities [68]. The study of van Duijn et al. [71] observed four EOPD patients in a consanguineous family from a genetically isolated population in the southwestern region of the Netherlands. Manifestation of symptoms occurred before 40 years and the progression of PD was slow. Patients presented two of three major symptoms: resting tremor, bradykinesia or muscular rigidity. Two individuals presented psychiatric symptoms and one showed the presence of psychotic episodes. Neither patient showed atypical features indicating involvement of additional neurological systems. These results suggest that *DJ-1* is an important locus for EOPD, which was confirmed by other studies of Bonifati et al. [72]. However, the study of Ibanez et al. [73] does not indicate that any mutations in this gene are common for EOPD.

HTRA2 (also known as OMI) is a mitochondrial serine protease encoded by *HTRA2* gene. During stimulation an apoptosis, HTRA2 is released from the mitochondria into the cytosol where it binds to inhibitor of apoptotic proteins (IAPs). It holds inhibitory activity towards caspases and activates proteolytic activity of this serine protease [74, 75]. HTRA2 is phosphorylated by PINK1 what modulates its proteolytic activity and increases cellular resistance of cells to mitochondrial stress [76]. The study of Whitworth et al. [77] suggests that there is genetical interaction between both of them, PINK1 and HTRA2.

Mutations in the *HTRA2* gene were found in family with an autosomal dominant manner. One of the missense mutation (G399S) was found in FPD, but the other (A141S), was more frequently detected in cases than in controls.

These results recognize second mutation as a risk factor. Furthermore, this A141S mutation leads to a decrease in protease function [59, 75]. Clinical symptoms include typical PD features (bradykinesia, tremor and muscular rigidity) and all patients responded well to L-dopa therapy. However, a study of Simon-Sanchez and Singleton [78] on a larger population found that both *HTRA2* variations were present in controls.

LRRK2 is a protein encoded by *LRRK2*, which is a serine/threonine kinase containing a conserved mitogen-activated protein kinase kinase kinase (MAPKKK), a Roc family GTPase domain [79, 80]. Overexpression of mutated protein LRRK2 induces neuronal death (initially blocked by caspase inhibitors) through Apaf1 induced apoptosis [81]. Many mutations lead to increase of the LRRK2 kinase activity, what is responsible for neurotoxicity *in vitro* [82]. The study of Niu et al. [83] shows that mutant form of G2019S *LRRK2*, can cause defects in the morphology and dynamics of mitochondria in cortical neurons. Endogenous LRRK2 interacts with the mitochondrial fission factor Dynamin like protein 1 (DLP1) and leads to translocation of DLP1 into the mitochondrial matrix. Moreover, overexpression of LRRK2 leads to increased ROS levels in cells.

The mutations of *LRRK2* gene are mostly associated with FPD. Paisan-Ruiz et al. [84] described four families from Spain and one family from United Kingdom with autosomal dominant PD. Mean age of onset was 65 years and characteristic features included milder progression with excellent response to low doses of L-dopa. Most of the patients presented unilateral leg or hand tremor and no cognitive impairment. The report of Healy et al. [85] shows that the common mutation G2019S occurs in 1% of patients with SPD and in 4% of patients with hereditary PD. The risk of PD for a person who inherits the G2019S *LRRK2* mutation was 28% at age 59 years, but 74% at 79 years.

## **DYSFUNCTION OF SYNAPTIC VESICLES IN PARKINSON'S DISEASES**

Synaptic transport, including exo- and endocytosis along with vesicle trafficking is a crucial mechanism to efficient neuronal function. The process starts with exocytosis at the presynaptic axon terminal site, when neurotransmitters are released from synaptic vesicles [86]. To enable further signal transmission, the neurotransmitters have to be recycled through endocytosis. Both processes are of extreme importance to synaptic efficiency,

hence they require complex and thorough regulation. It has been proved that upon periods of high demand regulatory proteins involved in vesicle cycle are being re-used.

However, this recycling, may be the cause of toxicity due to related protein damage [87, 88]. In this way, synaptic vesicle trafficking has been linked to neurodegeneration - main cause of PD [89].

Recently, Deng et al. [90] suggested *TMEM230* as a site of mutations corresponding with risk of PD development. Characteristics of *TMEM230* encoded product still remains limited. The gene however, comprises 5 exons, and its mRNA may be spliced into four different variants. TMEM230, a transmembrane protein is suggested to be composed of two  $\alpha$ -helical domains and two segments of N- and C-terminal sites reaching into the cytosol [91, 92]. Study of European family, in which 15 individuals suffered from PD led to identification of four mutations including one missense at said site, and it has been found in four affected family members [90]. The presence of *TMEM230* mutations was observed also in individuals of Chinese origin, however they have been linked only to FPD, excluding sporadic cases. Deng et al. [90] performed an immunohistochemical staining in order to map TMEM230 to its cellular localization. Their findings were similar in both mouse and human models localizing TMEM230 in neuronal synaptic vesicles, also in dopaminergic neurons in substantia nigra. Co-staining with TMEM230 and subcellular organelle markers reveled co-localization of TMEM230 with perinuclear clusters, secretory and synaptic vesicles indifferently of presence of PD associated mutations. Additionally, they proved TMEM230's contribution to reserve vesicle pool recovery through exocytosis subsequent to neuronal signal transduction [93]. This retrieval is mostly regulated by RAB5A, a member of RAB GTPase family, mediating early endosome formation [94]. Co-localization of TMEM230 and RAB5A has been observed in early endosomes. Furthermore, Deng et al. [90] suggest TMEM230 to be a synaptic vesicle trafficking mediator rather than neurotransmitter uptake regulator. This suggestion is based on smaller protein size and fewer transmembrane segments when compared with proteins related to neurotransmitter release. The live imaging analysis of vesicle movement proved that all four *TMEM230* mutations correlate with slower vesicle flow and reduction in transport speed in comparison to wild-type variant. Ultimately, studied individuals carrying mutations at TMEM230 locus, developed typical, LB associated PD, usually responsive to L-dopa treatment with main symptoms including: resting tremor and bradykinesia. What is more, the onset of the disease occurred between the age of 48 and 85 years old and concerned only cases of FPD. Although

*TMEM230* is the first gene, which product has been proved to be of significance in synaptic vesicle regulation, other, already identified genes have been suggested to correlate with PD pathobiology not only via known mechanisms, but also through its potential links to synaptic transport.

ASN, another protein seems to be correlated with synaptic vesicle transport through its interactions with cytoskeletal proteins [95, 96]. Moreover, Mak et al. [97] suggested SNCA contribution to age-related synaptic impairment studied *in vivo* on mouse models. Also, it has been proved that ASN negatively regulates synaptic vesicle size through lipid metabolism modulation upon periods of low neuronal activity.

It may even hold the capacity to inhibit synaptic vesicle formation entirely [98, 99]. Furthermore, Burre et al. [100] provided evidence for ASN contribution to synaptic vesicle trafficking. They found that ASN binds directly to synaptobrevin-2, one of SNARE proteins, and is essential to SNARE complex formation that occurs upon synaptic activity both *in vitro* and *in vivo*. Additionally, different extents of ASN overexpression induce different cellular responses. While toxicity is associated with immense SNCA over-expression, milder upregulation results in changes in synaptic transmission including vesicle trafficking and inhibition of neurotransmitter release [101]. Clinically, changes at SNCA site correspond with usual parkinsonism symptoms along with psychiatric manifestations such as hallucinations or paranoia [102]. Moreover, the inheritance tends to be autosomal dominant [103].

Another gene already linked to PD pathogenesis is LRRK2. LRRK2 is a large, multi-domain protein located in substantia nigra and striatum, thus suggesting its contribution to neuronal function [104-106]. Its impact on synaptic vesicle trafficking has been proved by co-localization of LRRK2 and Rab5b on endocytic vesicles [107, 108]. Moreover, lack of kinase activity in LRRK2 leads to depletion of endocytic vesicles thus undermining reserve vesicle pool recovery [109]. Mutations in *LRRK2* gene are associated with cases of FPD inherited through autosomal dominant manner [110-111]. Patients carrying those mutations usually tend to respond well to L-dopa treatment [112]. Apart from classic parkinsonism symptoms, other PD manifestations include gliosis in substantia nigra and secondary motor complications with rare dementia and cognitive impairment [113, 114]. LRRK2 associated PD is characterized by onset between the age of 50 and 65 and relatively slow progression of the disease [113, 115].

Therefore, recent research focuses on thorough study of genes already linked to risk of PD, rather than identifying novel mutations. Especially strong interest lies within broadening the knowledge on protein interactions and role

of changes proteins in vesicle trafficking and synaptic transmission, which are regarded as crucial in PD pathogenesis and seem to mediate the onset of the disease.

## **L-DOPA THERAPY EFFECTS IN PARKINSON'S DISEASE**

The most common agent used in the treatment of PD patients is L-dihydroxyphenylalanine (L-dopa). Literature data indicated that L-dopa therapy may induce side effects, lead to toxicity and even accelerate the progression of the disease. Long-term L-dopa treatment is associated with gradual efficacy reduction (occurring after 2-5 years), and also fluctuations or even enhancement of dyskinesias [14].

CNS neurons, because of their ability to storage excessive L-dopa compounds may function as buffering levels. However, PD progression and subsequent neuronal loss decreases brain storage capacity, causing fluctuations of L-dopa levels in the vicinity of dopaminergic neurons and thus triggering side effects development and contributing to poorer disease control. Inhibition (entacapone, tolcapone, carbidopa, benserazide, selegiline) of L-dopa peripheral metabolism reduces "off" phase, but does not protect from dyskinesias development [116, 117].

The literature data indicate that L-dopa therapy in PD sufferers may induce oxidative stress, and increase the inflammatory markers levels, and lead to abnormal biothiols metabolism (homocysteine, cysteine, methionine). ROS, generated as a result of auto-oxidation of DA and its precursor L-dopa, may induce PD progression. ROS contribute to PD through decreasing mitochondrial ATP production and impaired cell death mechanisms such as apoptosis and/or autophagy [14].

The hypothesis regarding oxidative stress as an important mechanism in dopaminergic neuron degeneration is supported by recent data on oxidative stress. It has been reported that extensive lipid peroxidation, increased oxidation of proteins, and overall oxidative DNA damage are observed *postmortem* in brain tissue of PD sufferers [14, 118, 119].

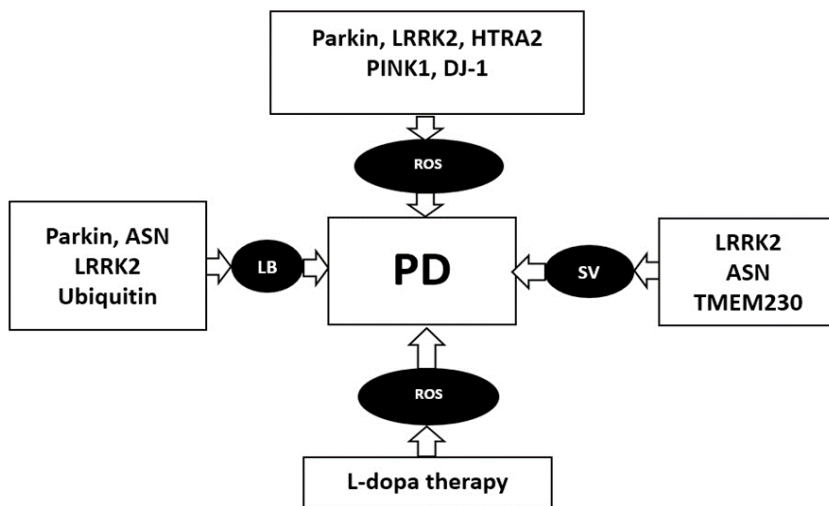


Figure 1. Role of molecular factors in pathogenesis of Parkinson's disease. PD- Parkinson's disease, ASN- alpha-synuclein, ROS- generation of reactive oxygen species, LB- formation of Lewy bodies, SV- dysfunction of synaptic vesicles.

## CONCLUSION

Improvement of diagnostics of CNS degenerative diseases (including PD) is a topic of major interest worldwide. To achieve this aim, an extensive research has been carried out in order to identify biomarkers able to provide insight into the risk of disease development, its progress and potential treatment response (Figure 1). Currently, complement diagnostic tests for PD including: PARK genetic variants and their protein products analysis, such as Parkin, ASN, DJ-1, PINK1, LRRK2, are suggested to allow earlier diagnosis. Consequently, progress in effective diagnostics could enable earlier introduction of pharmacotherapy.

Main factors regarded responsible for manifestation of PD are genetic mutations. Several PARK genes identified thus far, may be associated with the modulation of the risk of PD development. The state of knowledge regarding these genes variants. Alterations in *SNCA* and *PRKN* genes are thoroughly described, understanding of others such as *HTRA2*, remains poor.

Elucidation of PARK gene interaction mechanisms and other molecular factors (LB, synaptic vesicles) related to pathogenesis of PD could potentially

define new causes of selective damage to dopaminergic neurons in the course of PD.

## REFERENCES

- [1] Jenner, P., Sheehy, M. and Marsden, C. D. (1983). Noradrenaline and 5-hydroxytryptamine modulation of brain dopamine function: implications for the treatment of Parkinson's disease. *British Journal of Clinical Pharmacology*, 15, Suppl 2, 277-89.
- [2] Goldstein, D. S., Holmes, C. and Sharabi, Y. (2003). Plasma levels of catechols and metanephrines in neurogenic orthostatic hypotension. *Neurology*, 60, 1327-32.
- [3] Schapira, A. H. (2005). Present and future drug treatment for Parkinson's disease. *Journal of Neurology, Neurosurgery, and Psychiatry*, 76, 1472-8.
- [4] Dzięwulska, D. and Rafałowska, J. (2005). Rola zaburzeń przestrzennej budowy białek w patomechanizmie chorób układu pozapiramidowego. *Polish Journal of Neurology and Neurosurgery*, 39, 397-404. In Polish.
- [5] Dawson, T. M. and Dawson, V. L. (2003). Molecular pathways of neurodegeneration in Parkinson's disease. *Science*, 302, 819-22.
- [6] Dauer, W. and Przedborski, S. (2003). Parkinson's disease: mechanisms and models. *Neuron*, 39, 889-909.
- [7] Olanow, C.W. (2008). Levodopa/dopamine replacement strategies in Parkinson's disease-future directions. *Movement Disorders*, 23, Suppl. 3, S613-22.
- [8] Ikemura, M., Saito, Y., Sengoku, R., Sakiyama, Y., Hatsuta, H., Kanemaru, K., Sawabe, M., Arai, T., Ito, G., Iwatsubo, T., Fukayama, M. and Murayama, S. (2008). Lewy body pathology involves cutaneous nerves. *Journal of Neuropathology and Experimental Neurology*, 67, 945-53.
- [9] Wakabayashi, K., Takahashi, H., Takeda, S., Ohama, E. and Ikuta, F. (1988). Parkinson's disease: the presence of Lewy bodies in Auerbach's and Meissner's plexuses. *Acta Neuropathologica*, 76, 217-21.
- [10] Ehringer, H. and Hornykiewicz, O. (1960). Distribution of noradrenaline and dopamine (3-hydroxytyramine) in the human brain and their behavior in diseases of the extrapyramidal system. *Klinische Wochenschrift*, 38, 1236-9.



- [11] Lücking, C. B., Abbas, N., Dürr, A., Bonifati, V., Bonnet, A. M., de Broucker, T., De Michele, G., Wood, N. W., Agid, Y. and Brice, A. (1998). Homozygous deletions in parkin gene in European and North African families with autosomal recessive juvenile parkinsonism. The European Consortium on Genetic Susceptibility in Parkinson's Disease and the French Parkinson's Disease Genetics Study Group. *Lancet*, 352, 1355-6.
- [12] Abbas, N., Lücking, C. B., Ricard, S., Dürr, A., Bonifati, V., De Michele, G., Bouley, S., Vaughan, J. R., Gasser, T., Marconi, R., Broussolle, E., Brefel-Courbon, C., Harhangi, B. S., Oostra, B. A., Fabrizio, E., Böhme, G. A., Pradier, L., Wood, N. W., Filla, A., Meco, G., Deneffe, P., Agid, Y. and Brice, A. (1999). A wide variety of mutations in the parkin gene are responsible for autosomal recessive parkinsonism in Europe. French Parkinson's Disease Genetics Study Group and the European Consortium on Genetic Susceptibility in Parkinson's Disease. *Human Molecular Genetics*, 8, 567-74.
- [13] Bouhaddi, M., Vuillier, F., Fortrat, J. O., Cappelle, S., Henriët, M. T., Rumbach, L. and Regnard, J. (2004). Impaired cardiovascular autonomic control in newly and long-term-treated patients with Parkinson's disease: involvement of L-dopa therapy. *Autonomic Neuroscience*, 116, 30-8.
- [14] Dorszewska, J., Prendecki, M., Lianeri, M. and Kozubski, W. (2014). Molecular Effects of L-dopa Therapy in Parkinson's Disease. *Current Genomics*, 15, 11-7.
- [15] Hyun, D. H., Lee, M., Hattori, N., Kubo, S., Mizuno, Y., Halliwell, B. and Jenner, P. (2002). Effect of wild-type or mutant Parkin on oxidative damage, nitric oxide, antioxidant defenses, and the proteasome. *Journal of Biological Chemistry*, 277, 28572-7.
- [16] Walinda, E., Morimoto, D., Sugase, K. and Shirakawa M. (2016). Dual function of phosphoubiquitin in activation of parkin. *Journal of Biological Chemistry*, pii: jbc.M116.728600 (Epub ahead of print).
- [17] Vergara, D., Ferraro, M. M., Cascione, M., Del Mercato, L. L., Leporatti, S., Ferretta, A., Tanzarella, P., Pacelli, C., Santino, A., Maffia, M., Cocco, T., Rinaldi, R. and Gaballo A. (2015). Cytoskeletal alterations and biomechanical properties of parkin-mutant human primary fibroblasts. *Cell Biochemistry and Biophysics*, 71, 1395-404.
- [18] Stevens, D. A., Lee, Y., Kang, H. C., Lee, B. D., Lee, Y. I., Bower, A., Jiang, H., Kang, S. U., Andrabi, S. A., Dawson, V. L., Shin, J. H. and Dawson, T. M. (2015). Parkin loss leads to PARIS-dependent declines in

- mitochondrial mass and respiration. *Proceedings of the National Academy of Sciences of the United States of America*, 112, 11696-701.
- [19] Büeler, H. (2009). Impaired mitochondrial dynamics and function in the pathogenesis of Parkinson's disease. *Experimental Neurology*, 218, 235-46.
- [20] Dawson, T. M. and Dawson, V. L. (2014). Parkin plays a role in sporadic Parkinson's disease. *Neurodegenerative Diseases*, 13, 69-71.
- [21] Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura Y, Minoshima, S., Yokochi, M., Mizuno, Y. and Shimizu, N. (1998). Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature*, 392, 605-8.
- [22] Lücking, C. B., Dürr, A., Bonifati, V., Vaughan, J., De Michele, G., Gasser, T., Harhangi, B. S., Meco, G., Denèfle, P., Wood, N. W., Agid, Y. Brice, A., French Parkinson's Disease Genetics Study Group and European Consortium on Genetic Susceptibility in Parkinson's Disease. (2000). Association between early-onset Parkinson's disease and mutations in the parkin gene. *New England Journal of Medicine*, 342, 1560-7.
- [23] Bonifati, V. (2014). Genetics of Parkinson's disease – state of the art. (2013). *Parkinsonism and Related Disorders*, 20S1, S23–8.
- [24] Mata, I. F., Lockhart, P. J. and Farrer, M. J. (2004). Parkin genetics: one model for Parkinson's disease. *Human Molecular Genetics*, 13, 127-33.
- [25] Półrolniczak, A., Dorszewska, J., Florczak, J., Owecki, M., Różycka, A., Rubiś, B., Marcinkowski, M., Osmola, K., Krahel, A., Lewandowski, L., Jagodziński, P. and Kozubski, W. (2010). Analysis of PARK2 gene mutation in sporadic Parkinson's disease. *Folia Neuropathologica*, 48, 314.
- [26] Fiesel, F. C., Caulfield, T. R., Moussaud-Lamodière, E. L., Ogaki, K., Dourado, D. F., Flores, S. C., Ross, O. A. and Springer, W. (2015). Structural and Functional Impact of Parkinson Disease-Associated Mutations in the E3 Ubiquitin Ligase Parkin. *Human Mutation*, 36, 774-86.
- [27] Wauer, T., Simicek, M., Schubert, A. and Komander, D. (2015). Mechanism of phospho-ubiquitin-induced PARKIN activation. *Nature*, 524, 370-4.
- [28] Zhang, Y., Gao, J., Chung, K. K., Huang, H., Dawson, V. L. and Dawson, T. M. (2000). Parkin functions as an E2-dependent ubiquitin–protein ligase and promotes the degradation of the synaptic vesicle-associated

- protein, CDCrel-1. *Proceedings of the National Academy of Sciences of the United States of America*, 97, 13354-9.
- [29] Kay, D. M., Moran, D., Moses, L., Poorkaj, P., Zabetian, C. P., Nutt, J., Factor, S. A., Yu, C. E., Montimurro, J. S., Keefe, R. G., Schellenberg, G. D. and Payami, H. (2007). Heterozygous parkin point mutations are as common in control subjects as in Parkinson's patients. *Annals of Neurology*, 61, 47-54.
- [30] Kay, D. M., Stevens, C. F., Hamza, T. H., Montimurro, J. S., Zabetian, C. P., Factor, S. A., Samii, A., Griffith, A., Roberts, J. W., Molho, E. S., Higgins, D. S., Gancher, S., Moses, L., Zarepari, S., Poorkaj, P., Bird, T., Nutt, J., Schellenberg, G. D. and Payami H. (2010). A comprehensive analysis of deletions, multiplications, and copy number variations in PARK2. *Neurology*, 75, 1189-94.
- [31] Khan, N. L., Graham, E., Critchley, P., Schrag, A. E., Wood, N. W., Lees, A. J., Bhatia, K. P. and Quinn, N. (2003). Parkin disease: a phenotypic study of a large case series. *Brain*, 126, 1279-92.
- [32] Bilgic, B., Bayram, A., Arslan, A. B., Hanagasi, H., Dursun, B., Gurvit, H., Emre, M. and Lohmann, E. (2012). Differentiating symptomatic Parkin mutations carriers from patients with idiopathic Parkinson's disease: Contribution of automated segmentation neuroimaging method. *Parkinsonism and Related Disorders*, 18, 562-6.
- [33] Dawson, T. M. and Dawson, V. L. (2010). The role of parkin in familial and sporadic Parkinson's disease. *Movement Disorders*, 25, S32-9.
- [34] Ross, J. M., Olson, L. and Coppotelli, G. (2015). Mitochondrial and ubiquitin proteasome system dysfunction in ageing and disease: two sides of the same coin? *International Journal of Molecular Sciences*, 16, 19458-76.
- [35] Atkin, G. and Paulson, H. (2014). Ubiquitin pathways in neurodegenerative disease. *Frontiers in Molecular Neuroscience* 7, 63.
- [36] Vernace, V. A., Schmidt-Glenewinkel, T. and Figueiredo-Pereira, M. E. Aging and regulated protein degradation: Who has the UPPER hand? *Aging Cell*, 6, 599-606.
- [37] Oczkowska, A., Kozubski, W., Lianeri, M. and Dorszewska, J. (2013). Mutations in PRKN and SNCA genes important for the progress of Parkinson's disease. *Current Genomics*, 14, 502-17.
- [38] Oczkowska, A., Kozubski, W. and Dorszewska, J. (2014). Alpha-synuclein in Parkinson's disease. *Przegląd Lekarski*, 71, 26-32. In Polish.
- [39] Lim, K. L., Dawson, V. L. and Dawson, T. M. (2006). Parkin-mediated lysine 63-linked polyubiquitination: a link to protein inclusions formation

- in Parkinson's and other conformational diseases? *Neurobiology of Aging*, 27, 524-9.
- [40] Lee, M. K., Stirling, W., Xu, Y., Xu, X., Qui, D., Mandir, A. S., Dawson, T. M., Copeland, N. G., Jenkins, N. A. and Price, D. L. (2002). Human alpha-synuclein-harboring familial Parkinson's disease-linked Ala-53 -> Thr mutation causes neurodegenerative disease with alpha-synuclein aggregation in transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America*, 99, 8968-73.
- [41] Schmid, A. W., Fauvet, B., Moniatte, M. and Lashuel, H. A. (2013). Alpha-synuclein post-translational modifications as potential biomarkers for Parkinson disease and other synucleinopathies. *Molecular and Cellular Proteomics*, 12, 3543-58.
- [42] Barrett, P. J. and Greenamyre, T. J. (2015). Post-translational modification of  $\alpha$ -synuclein in Parkinson's disease. *Brain Research*, 1628, 247-53.
- [43] Polymeropoulos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A. Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E. S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W. G., Lazzarini, A. M., Duvoisin, R. C., Di Iorio, G., Golbe, L. I. and Nussbaum, R. L. (1997). Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science*, 276, 2045-7.
- [44] Krüger, R., Kuhn, W., Müller, T., Woitalla, D., Graeber, M., Kösel, S., Przuntek, H., Epplen, J. T., Schöls, L. and Riess, O. (1998). Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. *Nature Genetics*, 18, 106-8.
- [45] Zarranz, J. J., Alegre, J., Gómez-Esteban, J. C., Lezcano, E., Ros, R., Ampuero, I., Vidal, L., Hoenicka, J., Rodriguez, O., Atarés, B., Llorens, V., Gomez Tortosa, E., del Ser, T., Muñoz, D. G. and de Yebenes, J. G. (2004). The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. *Annals of Neurology*, 55, 164-73.
- [46] Puschmann, A. (2013). Monogenic Parkinson's disease and parkinsonism: Clinical phenotypes and frequencies of known mutations. *Parkinsonism and Related Disorders*, 19, 407-15.
- [47] Mata, I. F., Shi, M., Agarwal, P., Chung, K. A., Edwards, K. L., Factor, S. A., Galasko, D. R., Ghingina, C., Griffith, A., Higgins, D. S., Kay, D. M., Kim, H., Leverenz, J. B., Quinn, J. F., Roberts, J. W., Samii, A., Snapinn, K. W., Tsuang, D. W., Yearout, D., Zhang, J., Payami, H. and Zabetian, C. P. (2010). A SNCA variant associated with Parkinson's disease and plasma  $\alpha$ -synuclein level. *Archives of Neurology*, 67, 1350-6.

- [48] Fuchs, J., Tichopad, A., Golub, Y., Munz, M., Schweitzer, K. J., Wolf, B., Berg, D., Mueller, J. C. and Gasser, T. (2008). Genetic variability in the SNCA gene influences alpha-synuclein levels in the blood and brain. *FASEB Journal*, 22, 1327-34.
- [49] Rosenbusch, K. E. and Kortholt, A. (2016). Activation mechanism of LRRK2 and its cellular functions in Parkinson's disease. *Parkinson's Disease*, 2016, 7351985.
- [50] Gardet, A., Benita, Y., Li, C., Sands, B. E., Ballester, I., Stevens, C., Korzenik, J. R., Rioux, J. D., Daly, M. J., Xavier, R. J. and Podolsky, D. K. (2010). LRRK2 is involved in the IFN-gamma response and host response to pathogens. *Journal of Immunology*, 185, 5577-85.
- [51] Kachergus, J., Mata, I. F., Hulihan, M., Taylor, J. P., Lincoln, S., Aasly, J., Gibson, J. M., Ross, O. A., Lynch, T., Wiley, J., Payami, H., Nutt, J., Maraganore, D. M., Czyzewski, K., Styczynska, M., Wszolek, Z. K., Farrer, M. J. and Toft, M. (2005). Identification of a novel LRRK2 mutation linked to autosomal dominant Parkinsonism: evidence of a common founder across European populations. *American Journal of Human Genetics*, 76, 672-80.
- [52] García, S., López-Hernández, L. B., Suarez-Cuenca, J. A., Solano-Rojas, M., Gallegos-Arreola, M. P., Gama-Moreno, O., Valdez-Anguiano, P., Canto, P., Dávila-Maldonado, L., Cuevas-García, C. F. and Coral-Vázquez, R. M. (2014). Low prevalence of most frequent pathogenic variants of six PARK genes in sporadic Parkinson's disease. *Folia Neuropathologica*, 52, 22-9.
- [53] Kay, D. M., Zabetian, C. P., Factor, S. A., Nutt, J. G., Samii, A., Griffith, A., Bird, T. D., Kramer, P., Higgins, D. S. and Payami, H. (2006). Parkinson's disease and LRRK2: frequency of a common mutation in U.S. Movement Disorder Clinics. *Movement Disorders*, 21, 519-23.
- [54] Uttara, B., Singh, A. V., Zamboni, P. and Mahajan, R. T. (2009). Oxidative Stress and Neurodegenerative Diseases: A Review of Upstream and Downstream Antioxidant Therapeutic Options. *Current Neuropharmacology*, 7, 65-74.
- [55] Shimura, H., Hattori, N., Kubo, S., Mizuno, Y., Asakawa, S., Minoshima, S., Shimizu, N., Iwai, K., Chiba, T., Tanaka, K. and Suzuki, T. (2000). Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. *Nature Genetics*, 25, 302-5.
- [56] Norris, K. L., Hao, R., Chen, L. F., Lai, C. H., Kapur, M., Shaughnessy, P. J., Chou, D., Yan, J., Taylor, J. P., Engelender, S., West, A. E., Lim, K. L. and Yao, T. P. (2015). Convergence of Parkin, PINK1, and  $\alpha$ -Synuclein

- on Stress-induced Mitochondrial Morphological Remodeling. *Journal of Biological Chemistry*, 290, 13862-74.
- [57] Farrer, M., Chan, P., Chen, R., Tan, L., Lincoln, S., Hernandez, D., Forno, L., Gwinn-Hardy, K., Petrucelli, L., Hussey, J., Singleton, A., Tanner, C., Hardy, J. and Langston, J. W. (2001). Lewy bodies and parkinsonism in families with parkin mutations. *Annals of Neurology*, 50, 293-300.
- [58] Dawson, T. M. (2006). Parkin and defective ubiquitination in Parkinson's disease. *Journal of Neural Transmission*, 70, Suppl., 209-13.
- [59] Schulte, C. and Gasser, T. (2011). Genetic basis of Parkinson's disease: inheritance, penetrance, and expression. *Application of Clinical Genetics*, 4, 67-80.
- [60] Bonifati, V., Lücking, C. B., Fabrizio, E., Periquet, M., Meco, G. and Brice, A. (2001). Three parkin gene mutations in a sibship with autosomal recessive onset parkinsonism. *Journal of Neurology, Neurosurgery and Psychiatry*, 71, 531-4.
- [61] Narendra, D. P., Jin, S. M., Tanaka, A., Suen, D. F., Gautier, C. A., Shen, J., Cookson, M. R. and Youle, R. J. (2010). PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. *PLOS Biology*, 8, e1000298.
- [62] Morais, V. A., Verstreken, P., Roethig, A., Smet, J., Snellinx, A., Vanbrabant, M., Haddad, D., Frezza, C., Mandemakers, W., Vogt-Weisenhorn, D., Van Coster, R., Wurst, W., Scorrano, L. and De Strooper, B. (2009). Parkinson's disease mutations in PINK1 result in decreased Complex I activity and deficient synaptic function. *EMBO Molecular Medicine*, 1, 99-111.
- [63] Bonifati, V., Rohe, C. F., Breedveld, G. J., Fabrizio, E., De Mari, M., Tassorelli, C., Tavella, A., Marconi, R., Nicholl, D. J., Chien, H. F., Fincati, E., Abbruzzese, G. and (and others). (2005). Early-onset parkinsonism associated with PINK1 mutations: frequency, genotypes, and phenotypes. *Neurology*, 65, 87-95.
- [64] Albanese, A., Valente, E. M., Romito, L. M., Bellacchio, E., Elia, A. E. and Dallapiccola, B. (2005). The PINK1 phenotype can be indistinguishable from idiopathic Parkinson disease. *Neurology*, 64, 1958-60.
- [65] Toft, M., Myhre, R., Pielsticker, L., White, L. R., Aasly, J. O. and Farrer, M. J. (2007). PINK1 mutation heterozygosity and the risk of Parkinson's disease. *Journal of Neurology, Neurosurgery and Psychiatry*, 78, 82-4.
- [66] Clements, C. M., McNally, R. S., Conti, B. J., Mak, T. W. and Ting, J. P. (2006). DJ-1, a cancer and Parkinson's disease-associated protein, stabilizes the antioxidant transcriptional masterregulator Nrf2.

- Proceedings of the National Academy of Sciences of the United States of America*, 103, 15091-6.
- [67] Chen, J., Li, L. and Chin, L. S. (2010). Parkinson disease protein DJ-1 converts from a zymogen to a protease by carboxyl-terminal cleavage. *Human Molecular Genetics*, 19, 2395-408.
- [68] Björkblom, B., Adilbayeva, A., Maple-Grødem, J., Piston, D., Ökvist, M., Xu, X. M., Brede, C., Larsen, J. P. and Møller, S. G. (2013). Parkinson disease protein DJ-1 binds metals and protects against metal-induced cytotoxicity. *Journal of Biological Chemistry*, 288, 22809-20.
- [69] Gao, H., Yang, W., Qi, Z., Lu, L., Duan, C., Zhao, C. and Yang, H. (2012). DJ-1 protects dopaminergic neurons against rotenone-induced apoptosis by enhancing ERK-dependent mitophagy. *Journal of Molecular Biology*, 423, 232-48.
- [70] Takahashi-Niki, K., Niki, T., Taira, T., Iguchi-Ariga, S. M. and Ariga, H. (2004). Reduced anti-oxidative stress activities of DJ-1 mutants found in Parkinson's disease patients. *Biochemical and Biophysical Research Communications*, 320, 389-97.
- [71] van Duijn, C. M., Dekker, M. C., Bonifati, V., Galjaard, R. J., Houwing-Duistermaat, J. J., Snijders, P. J., Testers, L., Breedveld, G. J., Horstink, M., Sandkuijl, L. A., van Swieten, J. C., Oostra, B. A. and Heutink, P. (2001). PARK7, a novel locus for autosomal recessive early-onset parkinsonism, on chromosome 1p36. *American Journal of Human Genetics*, 69, 629-34.
- [72] Bonifati, V., Breedveld, G. J., Squitieri, F., Vanacore, N., Brustenghi, P., Harhangi, B. S., Montagna, P., Cannella, M., Fabbrini, G., Rizzu, P., van Duijn, C. M., Oostra, B. A., Meco, G. and Heutink, P. (2002). Localization of autosomal recessive early-onset parkinsonism to chromosome 1p36 (PARK7) in an independent dataset. *Annals of Neurology*, 51, 253-56.
- [73] Ibanez, P., De Michele, G., Bonifati, V., Lohmann, E., Thobois, S., Pollak, P., Agid, Y., Heutink, P., Durr, A., Brice, A. and French Parkinson's Disease Genetics Study Group. (2003). Screening for DJ-1 mutations in early onset autosomal recessive parkinsonism. *Neurology*, 61, 1429-31.
- [74] Martins, L. M., Turk, B. E., Cowling, V., Borg, A., Jarrell, E. T., Cantley, L. C. and Downward, J. (2003). Binding Specificity and Regulation of the Serine Protease and PDZ Domains of HtrA2/Omi. *The Journal of Biological Chemistry*, 278, 49417-27.
- [75] Strauss, K. M., Martins, L. M., Plun-Favreau, H., Marx, F. P., Kautzmann, S., Berg, D., Gasser, T., Wszolek, Z., Müller, T., Bornemann, A.,

- Wolburg, H., Downward, J., Riess, O., Schulz, J. B. & Krüger, R. (2005). Loss of function mutations in the gene encoding Omi/HtrA2 in Parkinson's disease. *Human Molecular Genetics*, 14, 2099-111.
- [76] Plun-Favreau, H., Klupsch, K., Moiso, N., Gandhi, S., Kjaer, S., Frith, D., Harvey, K., Deas, E., Harvey, R. J., McDonald, N., Wood, N. W., Martins, L. M. & Downward, J. (2007). The mitochondrial protease HtrA2 is regulated by Parkinson's disease-associated kinase PINK1. *Nature Cell Biology*, 9, 1243-52.
- [77] Whitworth, A. J., Lee, J. R., Ho, V. M., Flick, R., Chowdhury, R. and McQuibban, G. A. (2008). Rhomboid-7 and HtrA2/Omi act in a common pathway with the Parkinson's disease factors Pink1 and Parkin. *Disease Models and Mechanisms*, 1, 168-74.
- [78] Simon-Sanchez, J. and Singleton, A. B. (2008). Sequencing analysis of OMI/HTRA2 shows previously reported pathogenic mutations in neurologically normal controls. *Human Molecular Genetics*, 17, 1988-93.
- [79] Gandhi, P. N., Chen, S. G. and Wilson-Delfosse, A. L. (2009). Leucine-rich repeat kinase 2 (LRRK2): a key player in the pathogenesis of Parkinson's disease. *Neuroscience Research*, 87, 1283-95.
- [80] Biskup, S. and West, A. B. (2009). Zeroing in on LRRK2-linked pathogenic mechanisms in Parkinson's disease. *Biochimica et Biophysica Acta*, 1792, 625-33.
- [81] Iaccarino, C., Crosio, C., Vitale, C., Sanna, G., Carri, M. T. and Barone, P. (2007). Apoptotic mechanisms in mutant LRRK2-mediated cell death. *Human Molecular Genetics*, 16, 1319-26.
- [82] Gloeckner, C. J., Kinkl, N., Schumacher, A., Braun, R. J., O'Neill, E., Meitinger, T., Kolch, W., Prokisch, H. and Ueffing, M. (2006). The Parkinson disease causing LRRK2 mutation I2020T is associated with increased kinase activity. *Human Molecular Genetics*, 15, 223-32.
- [83] Niu, J., Yu, M., Wang, C. and Xu, Z. (2012). Leucine-rich repeat kinase 2 disturbs mitochondrial dynamics via Dynamin-like protein. *Journal of Neurochemistry*, 122, 650-58.
- [84] Paisan-Ruiz, C., Jain, S., Evans, E. W., Gilks, W. P., Simon, J., van der Brug, M., Lopez de Munain, A., Aparicio, S., Gil, A. M., Khan, N., Johnson, J., Martinez, J. R., Nicholl, D., Carrera, I. M., Pena, A. S., de Silva, R., Lees, A., Marti-Masso, J. F., Perez-Tur, J., Wood, N. W. and Singleton, A. B. (2004). Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. *Neuron*, 44, 595-600.
- [85] Healy, D. G., Falchi, M., O'Sullivan, S. S., Bonifati, V., Durr, A., Bressman, S., Brice, A., Aasly, A. J., Zabetian, C. P., Goldwurm, S., Ferreira,



- J. J., Tolosa, E., Kay, D. M., Klein, C., Williams, D. R., Marras, C., Lang, A. E., Wszolek, Z. K., Berciano, J., Schapira, A. H. V., Lynch, T., Bhatia, K. P., Gasser, T., Lees, A. J., Wood, N. W. on behalf of the International LRRK2 Consortium. (2008). Phenotype, genotype, and worldwide genetic penetrance of LRRK2-associated Parkinson's disease: A case-control study. *Lancet Neurology*, 7, 583-90.
- [86] Katz, B. and Miledi, R. (1968). The effect of local blockage of motor nerve terminals. *Journal of Physiology*, 199, 729-41.
- [87] Chandra, S., Gallardo, G., Fernandez-Chacon, R., Schluter, O. M. and Sudhof, T. C. (2005). Alpha-synuclein cooperates with CSPalpha in preventing neurodegeneration. *Cell*, 123, 383-96.
- [88] Uytterhoeven, V., Kuenen, S., Kasprowicz, J., Miskiewicz, K. and Verstreken, P. (2011). Loss of skywalker reveals synaptic endosomes as sorting stations for synaptic vesicle proteins. *Cell*, 145, 117-32.
- [89] Fernández-Chacón, R., Wölfel, M., Nishimune, H., Tabares, L., Schmitz, F., Castellano-Muñoz, M., Rosenmund, C., Montesinos, M. L., Sanes, J. R., Schneggenburger, R. and Südhof, T. C. (2004). The synaptic vesicle protein CSP alpha prevents presynaptic degeneration. *Neuron*, 42, 237-51.
- [90] Deng, H. X., Shi, Y., Yang, Y., Ahmeti, K. B., Miller, N., Huang, C., Cheng, L., Zhai, H., Deng, S., Nuytemans, K., Corbett, N. J., Kim, M. J., Deng, H., Tang, B., Yang, Z., Xu, Y., Chan, P., Huang, B., Gao, X. P., Song, Z., Liu, Z., Fecto, F., Siddique, N., Foroud, T., Jankovic, J., Ghetti, B., Nicholson, D. A., Krainc, D., Melen, O., Vance, J. M., Pericak-Vance, M. A., Ma, Y. C., Rajput, A. H. and Siddique, T. (2016). Identification of TMEM230 mutations in familial Parkinson's disease. *Nature Genetics*, 48, 733-9.
- [91] Roy, A., Kucukural, A. and Zhang, Y. (2010). I-TASSER: a unified platform for automated protein structure and function prediction. *Nature Protocols*, 5, 725-38.
- [92] Källberg, M., Wang, H., Wang, S., Peng, J., Wang, Z., Lu, H. and Xu, J. (2012). Template-based protein structure modeling using the RaptorX web server. *Nature Protocols*, 7, 1511-22.
- [93] Sudhof, T. C. (2004). The synaptic vesicle cycle. *Annual Review of Neuroscience*, 27, 509-47.
- [94] Bucci, C., Parton, R. G., Mather, I. H., Stunnenberg, H., Simons, K., Hoflack, B. and Zerial, M. (1992). The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway. *Cell*, 70, 715-28.

- [95] Kahle, P. J., Neumann, M., Ozmen, L., Muller, V., Jacobsen, H., Schindzielorz, A., Okochi, M., Leimer, U., van Der Putten, H., Probst, A., Kremmer, E., Kretschmar, H. A. and Haass, C. (2000). Subcellular localization of wild-type and Parkinson's disease-associated mutant  $\alpha$ -synuclein in human and transgenic mouse brain. *Journal of Neuroscience*, 20, 6365–73.
- [96] Alim, M. A., Hossain, M. S., Arima, K., Takeda, K., Izumiyama, Y., Nakamura, M., Kaji, H., Shinoda, T., Hisanaga, S. and Ueda, K. (2002). Tubulin seeds alpha-synuclein fibril formation. *Journal of Biological Chemistry*, 277, 2112–7.
- [97] Mak, S. K., McCormack, A. L., Langston, J. W., Kordower, J. H. and Di Monte, D. A. (2009). Decreased alpha-synuclein expression in the aging mouse substantia nigra. *Experimental Neurology*, 220, 359–65.
- [98] Liscovitch, M., Czarny, M., Fiucci, G. and Tang, X. (2000). Phospholipase D: molecular and cell biology of a novel gene family. *Biochemical Journal*, 345, 401–15.
- [99] Cole, N. B., Murphy, D. D., Grider, T., Rueter, S., Brasaemle, D. and Nussbaum, R. L. (2002). Lipid droplet binding and oligomerization properties of the Parkinson's disease protein  $\alpha$ -synuclein. *Journal of Biology* 277, 6344–52.
- [100] Burre, J., Sharma, M., Tsetsenis, T., Buchman, V., Etherton, M. R. and Sudhof, T. C. (2010). Alpha-synuclein promotes SNARE-complex assembly in vivo and in vitro. *Science* 329, 1663–7.
- [101] Nemani, V. M., Lu, W., Berge, V., Nakamura, K., Onoa, B., Lee, M. K., Chaudhry, F. A., Nicoll, R. A. and Edwards, R. H. (2010). Increased expression of alpha-synuclein reduces neurotransmitter release by inhibiting synaptic vesicle reclustering after endocytosis. *Neuron*, 65, 66–79.
- [102] Farrer, M., Kachergus, J., Forno, L., Lincoln, S., Wang, D. S., Hulihan, M., Maraganore, D., Gwinn-Hardy, K., Wszolek, Z., Dickson, D. and Langston, J. W. (2004). Comparison of kindreds with parkinsonism and alpha-synuclein genomic multiplications. *Annals of Neurology*, 55, 174–9.
- [103] Farrer, M., Gwinn-Hardy, K., Muentner, M., DeVrieze, F. W., Crook, R., Perez-Tur, J., Lincoln, S., Maraganore, D., Adler, C., Newman, S., MacElwee, K., McCarthy, P., Miller, C., Waters, C. and Hardy, J. (1999). A chromosome 4p haplotype segregating with Parkinson's disease and postural tremor. *Human Molecular Genetics*, 8, 81–5.

- [104] Biskup, S., Moore, D. J., Celsi, F., Higashi, S., West, A. B., Andrabi, S. A., Kurkinen, K., Yu, S. W., Savitt, J. M., Waldvogel, H. J., Faull, R. L., Emson, P. C., Torp, R., Ottersen, O. P., Dawson, T. M. and Dawson, V. L. (2006). Localization of LRRK2 to membranous and vesicular structures in mammalian brain. *Annals of Neurology*, 60, 557-69.
- [105] Melrose, H. L., Kent, C. B., Taylor, J. P., Dachsel, J. C., Hinkle, K. M., Lincoln, S. J., Mok, S. S., Culvenor, J. G., Masters, C. L., Tyndall, G. M., Bass, D. I., Ahmed, Z., Andorfer, C. A., Ross, O. A., Wszolek, Z. K., Delldonne, A., Dickson, D. W. and Farrer, M. J. (2007). A comparative analysis of leucine-rich repeat kinase 2 (Lrrk2) expression in mouse brain and Lewy body disease. *Neuroscience*, 147, 1047-58.
- [106] Mata, I. F., Wedemeyer, W. J., Farrer, M. J., Taylor, J. P. and Gallo, K. A. (2006). LRRK2 in Parkinson's disease: Protein domains and functional insights. *Trends in Neurosciences*, 29, 286-93.
- [107] de Hoop, M. J., Huber, L. A., Stenmark, H., Williamson, E., Zerial, M., Parton, R. G. and Dotti, C. G. (1994). The involvement of the small GTP-binding protein Rab5a in neuronal endocytosis. *Neuron*, 13, 11-22.
- [108] Wucherpennig, T., Wilsch-Brauninger, M. and Gonzalez-Gaitan, M. (2003). Role of Drosophila Rab5 during endosomal trafficking at the synapse and evoked neurotransmitter release. *Journal of Cell Biology*, 161, 609-24.
- [109] Piccoli, G., Condcliffe, S. B., Bauer, M., Giesert, F., Boldt, K., De Astis, S., Meixner, A., Sarioglu, H., Vogt-Weisenhorn, D. M., Wurst, W., Gloeckner, C. J., Matteoli, M., Sala, C. and Ueffing, M. (2011). LRRK2 controls synaptic vesicle storage and mobilization within the recycling pool. *Journal of Neuroscience*, 31, 2225-37.
- [110] Zimprich, A., Muller-Myhsok, Farrer, M., Leitner, P., Sharma, M., Hulihan, M., Lockhart, P., Strongosky, A., Kachergus, J., Calne, DB., Stoessel, J., Uitti, R. J., Pfeiffer, R. F., Trenkwalder, C., Homann, N., Ott, E., Wenzel, K., Asmus, F., Hardy, J., Wszolek, Z. and Gasser, T. (2004). The PARK8 locus in autosomal dominant parkinsonism: confirmation of linkage and further delineation of the disease-containing interval. *American Journal of Human Genetics*, 74, 11-9.
- [111] Funayama, M., Hasegawa, K., Ohta, E., Kawashima, N., Komiyama, M., Kowa, H., Tsuji, S. and Obata, F. (2005). An LRRK2 mutation as a cause for the parkinsonism in the original PARK8 family. *Annals of Neurology*, 57, 918-21.

- 
- [112] Hasegawa, K. and Kowa, H. (1997). Autosomal dominant familial Parkinson disease: older onset of age, and good response to levodopa therapy. *European Neurology*, 38, 39-43.
- [113] Wszolek, Z. K., Pfeiffer, B., Fulgham, J. R., Parisi, J. E., Thompson, B. M., Uitti, R. J., Calne, D. B. and Pfeiffer, R. F. (1995). Western Nebraska family (family D) with autosomal dominant parkinsonism. *Neurology*, 45, 502-5.
- [114] Ross, O. A., Toft, M., Whittle, A. J., Johnson, J. L., Papapetropoulos, S., Mash, D. C., Litvan, I., Gordon, M. F., Wszolek, Z. K., Farrer, M. J. and Dickson, D. W. (2006). Lrrk2 and Lewy body disease. *Annals of Neurology*, 59, 388-93.
- [115] Nichols, W. C., Pankratz, N., Hernandez, D., Paisan-Ruiz, C., Jain, S., Halter, C. A., Michaels, V. E., Reed, T., Rudolph, A., Shults, C. W., Singleton, A. and Foroud, T. (2005). Genetic screening for a single common LRRK2 mutation in familial Parkinson's disease. *Lancet*, 365, 410-12.
- [116] Pahwa, R., Factor, S. A., Lyons, K. E., Ondo, W. G., Gronseth, G., Bronte-Stewart, H., Hallett, M., Miyasaki, J., Stevens, J. and Weiner, W.J. (2006). Quality Standards Subcommittee of the American Academy of Neurology. Practice Parameter: treatment of Parkinson disease with motor fluctuations and dyskinesia (an evidence-based review): report of the Quality Standards Subcommittee of the American Academy of Neurology. *Neurology*, 66, 983-95.
- [117] Stocchi, F., Rascol, O., Kieburtz, K., Poewe, W., Jankovic, J., Tolosa, E., Barone, P., Lang, A. E. and Olanow, C. W. (2010) Initiating levodopa/carbidopa therapy with and without entacapone in early Parkinson disease: the STRIDE-PD study. *Annals of Neurology*, 68, 18-27.
- [118] Floor, E. and Wetzel, M.G. (1998). Increased protein oxidation in human substantia nigra pars compacta in comparison with basal ganglia and prefrontal cortex measured with an improved dinitrophenylhydrazine assay. *Journal of Neurochemistry*, 70, 268-75.
- [119] Kikuchi, A., Takeda, A., Onodera, H., Kimpara, T., Hisanaga, K., Sato, N., Nunomura, A., Castellani, R. J., Perry, G., Smith, M. A. and Itoyama, Y. (2002). Systemic increase of oxidative nucleic acid damage in Parkinson's disease and multiple system atrophy. *Neurobiology of Disease*, 9, 244-8.



## *Chapter 2*

# **STATINS AND YEAST POLYSACCHARIDES IN THE TREATMENT OF HYPERLIPIDEMIA AND LIVER STEATOSIS, ROLE OF AUTOPHAGY**

***T. P. Johnston<sup>1,\*</sup>, T. A. Korolenko<sup>2,†</sup> and N. P. Bgatova<sup>3,‡</sup>***

<sup>1</sup>Division of Pharmaceutical Sciences, School of Pharmacy,  
University of Missouri-Kansas City, Kansas City, MO, US

<sup>2</sup>Institute of Physiology and Basic Medicine, Novosibirsk, Russia

<sup>3</sup>Scientific Institute of Clinical and Experimental Lymphology,  
Novosibirsk, Russia

## **ABSTRACT**

Dyslipidemia is one of the major risk factors for the development of cardiovascular disease. Atherosclerosis resulting from hyperlipidemia causes many serious cardiovascular diseases. Statins are generally accepted as a treatment of choice for lowering of low-density lipoprotein (LDL) cholesterol, which reduces coronary heart disease morbidity and mortality. Since statin use can be associated with muscle problems and other adverse symptoms, non-adherence and discontinuation of statin

---

\* Corresponding Author: Thomas P. Johnston, Ph.D. Division of Pharmaceutical Sciences. School of Pharmacy. University of Missouri-Kansas City. Rm. 4243, HSB. 2464 Charlotte Street. Kansas City, MO 64108-2718, USA. P. 816-235-1624. F. 816-235-5779. Email: johnstont@umkc.edu.

† t.a.korolenko@physiol.ru.

‡ n\_bgatova@ngs.ru.

therapy often leads to inadequate control of hyperlipidemia and increased cardiovascular risk. Hence, there is a critical need to identify additional effective hypolipidemic agents that can be used either in combination with statins, or alone, if statins are not tolerated. Thus, newer hypolipidemic agents such as proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors, antisense oligonucleotides, cholesterol ester transfer protein (CETP) inhibitors, and microsomal triglyceride transfer protein (MTTP) inhibitors, as well as yeast polysaccharides (beta-glucans and mannans) and compounds derived from plants (phytopharmaceuticals) such as glucomannans, are being investigated.

Macrophages in atherosclerotic lesions are an important class of antigen-presenting cells, initiating an adaptive immune response to oxidized LDL (ox-LDL) antigens. When a statin drug (atorvastatin) was administered to mice prior to inducing acute lipemia with poloxamer 407 (P-407), lipid levels were reduced, but not back to baseline levels observed in controls. However, administration of beta-glucan (a yeast polysaccharide that stimulates macrophages) in combination with atorvastatin to P-407-induced hyperlipidemic mice resulted in normal lipid levels and demonstrated that beta-glucan was not only an effective adjuvant hypolipidemic agent when used with atorvastatin, but also that the combination was more effective than atorvastatin alone.

Recent investigations have revealed that dysfunction of autophagy (lypophagy) is involved in the progression of liver steatosis (in atherosclerosis) and chronic liver diseases such as alcoholic and nonalcoholic steatohepatitis and hepatocellular neoplasia. Hepatic steatosis disturbs autophagic proteolysis through the suppression of both autophagic induction and lysosomal function. Autophagy of lipids in the liver (lipophagy) may possibly have a protective role in the development of liver lipidosis commonly observed in atherosclerosis.

We investigated the effect of suppressing the functional activity of macrophages *in vivo* by the administration of gadolinium chloride ( $\text{GdCl}_3$ ), which removes a population of large macrophages in the liver and inhibits their rate of endocytosis. Pretreatment of P-407-induced hyperlipidemic mice with  $\text{GdCl}_3$  reduced the serum concentration of triglycerides and LDL-cholesterol at 24 hours, which, although speculative, may potentially be due to a hyper-compensatory increase in the endocytosis of lipids by other macrophage pools (e.g., lung and spleen).

In this chapter, we will discuss hyperlipidemia, its impact on the development of cardiovascular disease (CVD), and the use of statins, as well as both yeast polysaccharides and other miscellaneous newer and investigational non-statin agents, to treat hyperlipidemia. Autophagy and lipophagy will be discussed in view of lipid accumulation in the liver, as well as the gadolinium-induced model of macrophage depression in experimental biology and medicine. This model allows for the evaluation of the role of liver macrophages in various liver pathologies as it relates to

lipid turnover and lipid storage syndromes, which may give rise to new treatment paradigms for liver disease.

## **1. INTRODUCTION TO HYPERLIPIDEMIA AND ITS RELATION TO CARDIOVASCULAR DISEASE**

Atherosclerotic cardiovascular disease (CVD) is the major cause of mortality in industrialized countries. In the United States, CVD is still the leading cause of mortality, which accounted for about 24 percent of all deaths in 2014 [1]. In Italy, CVD was the primary cause in approximately 43% of all deaths during 1998 and 1999 [2]. Serum total cholesterol levels are directly related to coronary artery disease and low-density-lipoprotein cholesterol (LDL-C) is involved in the pathogenesis of the atherosclerotic changes that occur in the vascular wall [3]. Based on data collected from 2005 to 2008, over half of all adults in the United States have elevated cholesterol values and about one-third have elevated LDL-C levels [1]. The atherosclerotic process begins early in life [4, 5, 6] and may stop and resolve itself, however, it typically progresses to adult CVD. Genetic and environmental factors seem to contribute to the development of CVD [7].

Pharmacologic treatment of hyperlipidemia using statins for both primary and secondary prevention of cardiovascular disease is well accepted. However, most clinicians suggest changes in lifestyle (regular exercise, dietary control, avoiding high-stress environments for extended periods of time, *etc.*) to those patients to whom they prescribe statins. Primary prevention of cardiovascular heart disease is generally defined as treating patients with hyperlipidemia prior to active CVD (e.g., myocardial infarction secondary to atherosclerosis), whereas secondary prevention of CVD is defined as the treatment of hyperlipidemia after the detection of CVD and involves the application of interventions to prevent further progression of the CVD. While statins have the most convincing data for primary prevention of CVD, especially for higher risk patients, nevertheless, there are newer primary and adjunctive treatments that are being investigated to modify atherogenic plasma lipid profiles. Statin therapy is also currently recommended for secondary prevention of CVD in all patients with known cardiovascular disease or the risk equivalent [8]. For example, it is currently recommended that high-dose statins be initiated in patients with acute coronary syndrome [8]. For completeness sake, it should be mentioned that high-dose statin therapy has been associated with a slightly increased risk of the development of diabetes [9, 10].



While the use of non-statins to treat hyperlipidemia includes bile acid-binding resins, ezetimibe, fibrates, nicotinic acid, and omega-3 fatty acids, these agents will not be discussed in this chapter so that newer non-statin agents, some of which are still investigational, can be described. In particular, hypolipidemic agents such as proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors, antisense oligonucleotides, cholesteryl ester transfer protein (CETP) inhibitors, and microsomal triglyceride transfer protein (MTTP) inhibitors will be briefly described, but it is our intent in this chapter to focus on yeast polysaccharides and compounds derived from plants (phytopharmaceuticals) such as mannans, glucomannans, and beta-glucans. Some of these agents have very significant roles in terms of macrophage activation. Macrophage activation, in turn, may lead to a higher rate and extent of lipid endocytosis and, consequently, reductions in circulating lipids and lipoproteins. But, first we begin with statins and their use in treating hyperlipidemia, since this class of hypolipidemic agent was mentioned in the chapter title.

## **2. THERAPEUTIC AGENTS USED TO TREAT HYPERLIPIDEMIA**

### **a. Statins**

No individual statin has been proven superior at preventing coronary heart disease (CHD). In fact, at equivalent doses, all statins significantly reduce LDL cholesterol [11]. The primary target when treating hyperlipidemia based on guidelines developed in the U.S. and other countries is LDL-cholesterol, whether that involves the use of statins in a fixed-dose treatment algorithm or a treat-to-target treatment paradigm. The U.S. National Cholesterol Education Program, Adult Treatment Panel (NCEP-ATP) III guidelines suggest a treat-to-target approach and are, in general, more aggressive than guidelines developed by other countries [12]. It is important that patients be risk stratified with regard to their CVD risk and treated appropriately with statins while taking into consideration the ATP III guidelines and target goals, potential for adverse side effects (rhabdomyolysis), and the rather small chance of developing ‘statin intolerance/resistance.’ Studies have demonstrated that statin use in primary prevention of CVD benefits patients with the greatest baseline risk [8].

Various large meta-analyses have been conducted to ascertain the therapeutic value of using statins for primary prevention of CVD and have

shown mixed results when compared to placebo for all-cause mortality due to CVD [13, 14]. In summary, however, it is generally well-accepted that statin therapy can, and does, improve overall outcomes for premature development of coronary heart disease (CHD) when patients present as 'high-risk' based on scores estimated with validated prediction models, such as the Reynolds risk score analysis or the Framingham risk assessment software program [11].

In terms of using statins for the secondary prevention of CVD, all patients with a history of CVD benefit from statin therapy as mentioned above. In patients with a history of CVD, ATP III guidelines would suggest statin therapy to achieve an absolute LDL-C plasma concentration below 100 mg/dl, or even below 70 mg/dl depending on the total number of risk factors present for a given patient. Importantly, statins would appear to benefit patients with existing CHD regardless of baseline cholesterol levels or age [15, 16, 17], especially as it relates to the odds of experiencing future myocardial infarction and stroke. As Last et al. suggest [8], the optimal starting dose of a statin for the secondary prevention of CVD depends on the presence of acute coronary syndrome. In fact, following a review of the literature on statin use in the secondary prevention of CHD, Last et al. would further suggest initiation of a statin drug at a lower dose for patients with stable CHD, and reserve higher dose statin therapy for those patients with recent acute coronary syndrome [8].

## **b. Non-Statins from Natural Sources (Yeast Polysaccharides, Mannans, Glucomannan, and Beta-Glucans)**

As it pertains to non-statin compounds used to treat hyperlipidemia, the slightly water-soluble, wall-yeast polysaccharide known as zymosan has been shown to decrease atherogenic serum lipids in experimental lipemia induced in mice, although the hypolipidemic effects of zymosan, which is composed primarily of  $\beta$ -glucan and mannan, are still poorly understood [18]. The former component; namely,  $\beta$ -glucan, has previously been shown to exhibit a hypolipidemic effect in a well-documented mouse model known as the poloxamer 407 (P-407)-induced hyperlipidemic mouse model of atherogenesis [19, 20]. Thus, besides the use of the statin class of drugs for lowering LDL-cholesterol, biological response modifiers, such as the partially water-soluble  $\beta$ -1,3-glucans, may be used as adjunctive therapy with the statins. These natural polysaccharides are much less expensive than statin therapy and have no adverse side effects on skeletal muscle.

Mannan, which belongs to a class of immunomodulators of polysaccharide origin, has been shown to stimulate macrophages *in vivo* through its interaction with the mannose receptor [21-24]. As mentioned above, polysaccharides, unlike statins, are natural stimulators of macrophages, which cause the macrophages to increase their endocytic activity [25, 26]. Following endocytosis, these branched polysaccharides have the capacity to activate LDL and scavenger receptors and increase the uptake of atherogenic LDL-cholesterol, as well as other lipoproteins with modified chemical structures. It has also been suggested that excess cholesterol may potentially be removed from atherosclerotic plaque by activated macrophages [27].

Due to the fact that polysaccharides cause an increase in the activity of macrophages, they consequently promote an increase in the secretion of an enzyme called chitotriosidase by macrophages, which may then serve as a biomarker of macrophage stimulation/activation. In fact, chitotriosidase has been suggested to represent a new, non-lipid biomarker for the development of early atherosclerosis and has been demonstrated in individuals with established atherosclerosis [28-31]. By utilizing new biomarkers (e.g., chitotriosidase), clinicians will be able to identify those at risk for developing atherosclerosis when changes in serum lipid profiles are unremarkable and would otherwise not suggest a patient at risk for the development of coronary heart disease secondary to atherosclerosis. More importantly, these same biomarkers may provide insight on the effectiveness of various therapeutic (hypolipidemic) interventions, for example, the administration of standard hypolipidemic drugs used either as monotherapy (e.g., statins), or in combination with, various phytonutrients such as  $\beta$ -glucan, mannan, and other natural compounds.

Plant sterols have also been studied as cholesterol-lowering agents since the early 1950s [32]. Moreover, plant-derived sterols have now been unequivocally established as effective compounds with which to lower serum cholesterol [33-36]. In fact, similar to ezetimibe, plant sterols lower LDL-cholesterol concentrations by inhibiting cholesterol absorption from the intestine [37, 38].

Glucomannan fiber, obtained from *Amorphophallus konjac* tubers, has been documented to function as both a hypolipidemic [39-42] and hypoglycemic agent [41, 43, 44]. It has been suggested that glucomannans reduce serum cholesterol concentrations by decreasing the rate of cholesterol neosynthesis in the liver. In addition, glucomannan may decrease LDL-cholesterol without changing either high-density-lipoprotein cholesterol (HDL-C) levels or the extent of intestinal absorption of iron, calcium, copper, and zinc [40, 42].

Glucomannan swells in the presence of water. The resulting viscous gel delays gastric emptying time and consequently decreases the postprandial surge

in plasma glucose and insulin [45, 46]. A decrease in the postprandial insulin concentration suppresses hepatic cholesterol synthesis [47] possibly through a reduction in insulin-induced 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity [48]. If this mechanism of action is assumed to be accurate, then glucomannan's pharmacological response with regard to HMG-CoA reductase activity (inhibition) would be similar to that of statins. As with several plant-derived anti-hypercholesterolemic compounds (e.g., psyllium fiber), glucomannan appears to increase not only fecal weight [43], but also the excretion of bile acids into the feces [42, 49]. Since cholesterol is normally excreted into bile, the decrease in the plasma concentration of bile acids [49] and the subsequent excretion of bile acids into the feces is considered to represent a portion of the cholesterol-lowering mechanism of glucomannan [40, 50, 51].

Yoshida et al. investigated the anti-hypercholesterolemic effects of dietary supplementation with plant sterols and/or glucomannan to determine whether these supplements would both improve the overall lipid profile and modulate cholesterol biosynthesis in mildly hypercholesterolemic type II diabetic and non-diabetic subjects [52]. Eighteen non-diabetic individuals and 16 type II diabetic individuals studied in a randomized, crossover study consisting of four phases of 21 days, with each phase separated by a 28-day washout period [52]. Study subjects were supplemented with plant sterols (1.8 g/day), glucomannan (10 g/day), a combination of glucomannan and plant sterols, and a placebo, provided in the form of bars. Although overall plasma cholesterol concentrations were lowered ( $p < 0.05$ ) after combination treatment ( $4.72 \pm 0.20$  mmol/l) when compared to controls ( $5.47 \pm 0.18$  mmol/l), there was no statistically significant difference between plasma total cholesterol concentrations with glucomannan supplementation alone vs. controls [52]. However, plasma LDL-cholesterol concentrations were decreased ( $p < 0.05$ ) after glucomannan ( $3.16 \pm 0.14$  mmol/l) and combination treatments ( $2.95 \pm 0.16$  mmol/l) compared to control ( $3.60 \pm 0.16$  mmol/l). As an index of cholesterol biosynthesis, overall plasma lathosterol concentrations were lower ( $p < 0.05$ ) after the combination treatment compared to the plant sterol treatment, which supports the hypothesis that glucomannan functions, in part, by inhibiting cholesterol neosynthesis in the liver [52]. Thus, Yoshida et al. concluded that both glucomannan alone and a combination of glucomannan and plant sterols substantially improves plasma LDL-cholesterol concentrations in type II diabetic patients [52].

Martino et al. suggest that glucomannan may represent a rationale adjunct to diet therapy in primary prevention of CVD in high-risk hypercholesterolemic

children [53]. A fiber-rich diet that is low in fat and cholesterol is typically the initial treatment strategy in hypercholesterolemic children. The diet itself may decrease both plasma total cholesterol and LDL-cholesterol and may contribute to a decrease in the incidence of CVD [41, 54]. The type of dietary fiber used in the treatment of hypercholesterolemia is critical. Water-soluble fibers such as pectin, gums, and mixed-linked  $\beta$ -1,3- and 1,4-d-glucans are capable of significantly lowering the plasma cholesterol concentration [54, 55].

As mentioned above, glucomannan is a water-soluble fiber. When Martino et al. evaluated the anti-hypercholesterolemic effect of glucomannan in hypercholesterolemic children, the glucomannan-treated group showed decreased values in plasma total cholesterol and LDL-cholesterol relative to the control group after 8 weeks of dietary supplementation [53]. Interestingly, female children had a significantly greater reduction in total cholesterol (24% vs. 9%) and LDL-cholesterol (30% vs. 9%) than male children treated with the same glucomannan-supplemented diet [53]. They concluded that dietary supplementation with glucomannan may represent a rationale adjunct to diet therapy alone in the primary prevention of hyperlipidemia and future CVD in high-risk hypercholesterolemic children.

### **c. Miscellaneous Newer and/or Investigational Non-Statins Agents (MTTP, CETP, PCSK9 Inhibitors, and Antisense Oligonucleotides)**

Several newer and/or investigational non-statin agents have been evaluated that utilize different mechanisms of action to decrease cholesterol. However, this chapter is more focused on statins and non-statins derived from natural sources (e.g., yeast polysaccharides, mannans, glucomannan, and beta-glucans). Thus, only a brief review of some of these newer agents will be addressed. Antisense oligonucleotides (e.g., mipomersen) actually represents the complimentary DNA (cDNA) for the messenger RNA (mRNA) of the apolipoprotein B gene [56, 57, 58]. The cDNA oligonucleotide “hybridizes” with the mRNA species of interest and forms a cDNA-mRNA complex [57, 58]. Essentially, the mRNA cannot be translated into protein. Therefore, the decrease in apolipoprotein B-100 (apoB-100) protein decreases the availability for LDL synthesis, and consequently, plasma LDL is reduced [56, 57, 58]. The dosing regimen for mipomersen is weekly subcutaneous injections and this agent has only been approved to date for familial hypercholesterolemia.

The microsomal triglyceride transfer protein (MTTP) inhibitors are represented by lomitapide. MTTP normally transfers triglycerides to the endoplasmic reticulum where newly-synthesized apolipoproteins are added to make a chylomicron or VLDL particle [59]. It should be mentioned that cholesterol is also absorbed, subsequently esterified, and then packed into chylomicrons in the intestine or VLDL particles in the liver. The mechanism of action for lomitapide is to inhibit MTTP. Since MTTP is required for chylomicron and VLDL assembly and secretion, a decrease in their production interferes downstream in the formation of LDL-C [59, 60]. Thus, plasma LDL-C is reduced as a result. One drawback to the use of lomitapide is the significant adverse effect of increased aminotransferase levels and the potential for hepatotoxicity (hepatic steatosis that could progress to cirrhosis) [61]. Similar to the antisense oligonucleotide mipomersen, lomitapide has only been approved to date for treatment of familial hypercholesterolemia.

Proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors represent yet another potential treatment strategy for some patients that cannot take statins and that may suffer from familial hypercholesterolemia [60, 62, 63]. Briefly, PCSK9 is a member of the proteinase K family and binds to the LDL receptor resulting in its degradation [64]. Naturally, this action would decrease the uptake and metabolism of LDL from the blood, which subsequently results in an increase of LDL levels in the blood [62, 64]. The rationale behind the use of the PCSK9 inhibitor, which is itself a monoclonal antibody, is to recognize PCSK9, bind to it, and render PCSK9 inactive and incapable of destroying LDL receptors. Intact LDL receptors are then free to scavenge more LDL-C from the circulation and the blood levels of LDL-C are significantly reduced [60, 62, 64]. Alirocumab and evolocumab are two PCSK9 inhibitors (both are human monoclonal antibodies) approved in 2015 [65, 66, 67]. In Phase II trials, alirocumab was shown to decrease LDL-C by up to 73% [68], but it should be noted that both agents are extremely expensive and are reserved for those patients with either 1) familial hypercholesterolemia, 2) 'statin intolerance', or 3) patients simply unable to lower their cholesterol levels with statins and other hypolipidemic therapies. One recent article also reported that PCSK9 inhibitors reduce mortality, but increase neurocognitive events in hypercholesterolemia [69].

Lastly, another non-statin investigational class of compounds includes the cholesteryl ester transfer protein (CETP) inhibitors [70]. CETP, a glycoprotein synthesized in the liver, normally mediates the transfer of cholesteryl esters from larger subfractions of HDL (i.e., HDL<sub>2</sub>) to triglyceride-rich lipoproteins and LDL in exchange for a molecule of triglyceride [70]. Following HDL<sub>2</sub>'s

enrichment with triglycerides, it becomes more susceptible to catabolism by the liver [71]. Some examples of hypolipidemic agents in the CETP inhibitor category include torcetrapib, anacetrapib, dalcetrapib, and evacetrapib, although it should be mentioned that these agents are not without risk, since clinical trials with torcetrapib were halted in 2006 due to increased mortality [72]. When either these inhibitors are evaluated in laboratory animals, or a genetic mutation occurs in the gene that encodes for the synthesis of CETP in humans, the result is similar; specifically, greater levels of HDL and reduced levels of LDL-C, although there are conflicting reports as to whether this modulation in the levels of HDL and LDL-C that occur from a CETP mutation in humans leads to an increased or decreased risk of CVD. It must be mentioned that due to the failure of dalcetrapid and evacetrapib, there are numerous review articles appearing in the literature that do not support the rationale for the continued development of CETP inhibitors [73-76]. Nevertheless, the CETP inhibitors represent yet another strategy of interfering with cholesterol metabolism in an attempt to reduce LDL-C and increase HDL, although their clinical efficacy in the treatment of hypercholesterolemia remains in question.

We now turn our attention to the second focus of this chapter. Specifically, lipid accumulation in liver cells and the role of autophagy and lipophagy in handling excess lipids. Lastly, we shall conclude the chapter with a discussion on the gadolinium-induced model of liver macrophage depression in experimental biology and medicine.

### **3. LIPID ACCUMULATION IN LIVER CELLS, MECHANISMS, AND ROLE OF AUTOPHAGY AND LIPOPHAGY**

Non-alcoholic fatty liver disease includes several kinds of liver pathology which is characterized by accumulation of lipids in liver cells (steatosis). Steatosis in combination with hepatic inflammatory reaction is called steatohepatitis [77, 78]. The mechanisms associated with steatosis and steatohepatitis development are still not completely understood. It has been shown that cholesterol accumulation occurs in liver macrophages (Kupffer cells) enriched with scavenger receptors [79-82]. Autophagy in macrophages regulates the inflammasome and protects against liver injury. Accumulation of cholesterol has been shown in lysosomes of Kupffer cells with formation of cholesterol crystals inside of lysosomes, which activate inflammasomes. Activation of caspase-1 induces maturation of proinflammatory cytokines such

as interleukin-1-beta and IL-18 involved with inflammation. It has been suggested that disturbances in autophagy and decreased cholesterol efflux stimulates cholesterol crystallization in Kupffer cells and leads to hepatic inflammation [77].

Recently, several studies have identified that the activation of autophagy attenuates hepatic steatosis [79, 80, 81]. Three types of autophagy have been shown in the liver; namely, macroautophagy, chaperon-mediated autophagy, and microautophagy [83]. Decreased lipid catabolism, or an increase in lipid influx into the liver, or a combination of these two processes, results in intrahepatic accumulation of lipids, followed by steatosis or steatohepatitis. In general, lipid storage syndromes with simultaneous inflammation over a significant period of time result in liver fibrosis.

Autophagy in liver cells plays an important role in toxic liver damage induced by different hepatotoxic agents [84]. The involvement of autophagy in the pathogenesis of steatosis was suggested by the finding that this pathway mediates the breakdown of intracellular lipids in hepatocytes and may regulate the development of hepatic steatosis [85]. Moreover, autophagy is considered as a potential therapeutic target in atherosclerosis [86]. Autophagy in hepatocytes, liver macrophages, and stellate cells can regulate insulin sensitivity, hepatocellular injury, innate immunity, fibrosis, and carcinogenesis. Macrophage autophagy regulates the innate immune response in steatosis, and lipophagy regulates hepatocellular fat accumulation [85, 87]. Autophagy, being a novel selective pathway in lipid breakdown, has been termed 'lipophagy' [88].

In either Triton WR 1339 [89] or poloxamer 407 is used to induce a profound experimental hyperlipidemia (and disordered lipid metabolism) in mice, a possible mechanism for the development of steatosis includes hepatocyte injury, inflammation, and subsequent development of various degrees of fibrosis, especially if either agent is administered repeatedly to maintain sustained elevated serum lipids [90]. Excessive fatty acid influx to the liver can be associated with lipotoxicity and cellular stresses, which result in hepatocyte damage [91]. Autophagy is one of the most important factors regulating lipid storage syndrome development in liver cells [85, 92]. Statin therapy, in general, improves steatosis and abates cirrhosis development [93, 94]. Moreover, the treatment of the inflammatory component of the disease by Resolvin 1 (without statin therapy) reduced atherosclerotic lesion size and attenuated the formation of severe lesions without altering plasma cholesterol levels [95]. In contrast, agents of different origins known to stimulate macrophages [26, 96, 97] can be used to intentionally increase autophagy in macrophages so as to remove excess lipids in liver cells. Hypercholesterolemia



was attributed to the growing list of factors associated with aberrant cardiac mTOR signaling [98, 99]. mTOR, or the mammalian target of rapamycin, is a primary inducer of autophagy and plays a central role in integrating the cell's response to nutritional status [98, 99]. According to a review by De Meyer et al. [100] autophagy in macrophages is responsible for the clearance of dead cells and cell detritus, as well as increased cholesterol efflux from foam cells. This latter function, in general, represents a positive role of autophagy in cardiovascular pathology. At the same time defective autophagy in vascular smooth muscle cells accelerates senescence and promotes atherogenesis [101, 102]. Autophagy plays an important role in cardiac hypertrophy and heart failure, shown in the case of cathepsin L (a cysteine protease expressed in murine and human heart) deficiency [103, 104]. However, to date, there are conflicting reports about whether the role of autophagy by cardiomyocyte is a protective mechanism for relieving disease pathogenesis, or it is a process that enhances disease progression depending on the level of autophagocytic activity in cardiomyocytes. It was suggested that cardiomyocyte autophagy, which plays a role in controlling the hypertrophic response, can be a potential therapeutic target for cardiac hypertrophy [102] although these studies are still in progress.

#### **4. THE GADOLINIUM-INDUCED MODEL OF LIVER MACROPHAGE DEPRESSION IN EXPERIMENTAL BIOLOGY AND MEDICINE**

The mechanism of action and side effects of gadolinium-based contrast agents, widely used in magnetic resonance imaging, are being intensively investigated at the present time [105]. In rare cases, gadolinium agents can lead to the development of nephrogenic systemic fibrosis in humans (an acute adverse event) and significant presence of gadolinium in brain tissue. The deposition of gadolinium in individuals with normal renal function can be verified by histopathological analysis and is known as the “gadolinium storage condition” [106]. Several other complications following the use of gadolinium-based contrast agents have been described [107].

A few macrophage depression animal models have been developed and used in experimental biology and medicine. One of the models involves the intravenous administration of gadolinium chloride, first introduced by Hardonk et al. [108], and successfully used in later studies (see Table). Using this model, it is possible to elucidate the role of macrophages in different physiological

[109] and pathological processes, such as inflammation [110], immunological diseases, and toxicological pathology [111, 112, 113]. Some pathological processes are related to macrophages, and include situations where it might be necessary to suppress the increased macrophage activity with the help of  $\text{GdCl}_3$  [114, 115, 116]. This above model is easy to reproduce and it has been thoroughly studied and used by many authors in their *in vivo* [117-120] and *in vitro* [121] experiments. *In vitro*,  $\text{GdCl}_3$  revealed a dose-dependent effect on phagocytosis of cultured Kupffer cells, but only weak effects on cell viability and TNF-alpha production by these cells [121].

The positive aspect associated with this model is that administration of suitable doses of  $\text{GdCl}_3$  (in doses up to 20 mg/kg) selectively blocked the effector function of Kupffer cells, but did not cause toxicity to liver parenchymal cells and provides a basis for the establishment of an animal model for the study of Kupffer cell signaling in the liver [114]. Usynin et al. [116] have shown that Kupffer cell blockade by  $\text{GdCl}_3$  significantly decreased the clearance rate of oxidized [ $^{125}\text{I}$ ]-LDL from the blood by the liver (1.3-fold compared to control), but increased the clearance rate in the aorta (2.5-fold), heart (2-fold), lungs (1.6-fold), and kidney (1.3-fold). It was suggested that the accumulation of oxidized LDL in heart and aorta depends significantly on the functional state of the mononuclear phagocyte system in the liver.

A special characteristic of this model is the lysosomotropic effect of gadolinium chloride *in vivo* (i.e., the capacity for  $\text{GdCl}_3$  to be accumulated inside of lysosomes following intravenous administration). According to de Duve et al. [122], several compounds containing metals, such as cadmium, ferrum, cuprum, and lanthanoids, as well as  $\text{GdCl}_3$ , were included into the list of lysosomotropic agents.

Macrophage depression (induced by intravenous  $\text{GdCl}_3$ , 7-20 mg/kg) and macrophage stimulation (induced by intravenous zymosan, 100 mg/kg) have been studied as opposing processes in liver pathology [115, 120, 123]. It has been shown that  $\text{GdCl}_3$  treatment of rats reduced the rate of carbon particles phagocytosed at 24 and 48 h after a single intravenous dose. Decreased endocytic capacity of Kupffer cells was also confirmed by electron microscopy. Gadolinium chloride induced labilization of liver lysosomes based on an increase in the free activity of cathepsins B and L, although there was no change in the specific activity of these liver cysteine proteinases in Wistar rats 24 h after a single dose.

**Table. Macrophage depression effects in experimental pathology**

Experimental pathology	Effect	References
Intact Kunming mice, GdCl <sub>3</sub> (10-20 mg/kg, single)	Apoptosis of Kupffer cells and blocked the Kupffer cell effector function, decrease in CD68 expression and phagocytic activity. Hepatotoxicity was not observed.	Ding et al., 2003 [114]
Osteopetrotic (Csf1(op)/Csf1(op)) mice lacking functional M-CSF, reduced levels of Kupffer cells), rats (clodronate liposomes, reduced Kupffer cells); Cynomolgus macaques (human monoclonal antibody reducing the CD14 <sup>(+)</sup> CD16 <sup>(+)</sup> monocyte population, depleted KCs)	Increased serum levels of AST and creatine kinase associated with Kupffer cell reduction with no signs of hepatic or skeletal muscle injury.	Radi et al., 2011 [127]
Intact Kunming mice, GdCl <sub>3</sub> (40 mg/kg, single)	Caused both hepatotoxicity and Kupffer cell necrosis, an increased release of TNF, NO, and PGE <sub>2</sub> in the liver	Ding et al., 2003 [114]
Dimethylnitrosamine-induced liver fibrosis in rats	Suppression of collagen accumulation in liver cells (Sorafenib plus GdCl <sub>3</sub> )	Liu et al., 2015 [119]
Pig serum-induced rat liver fibrosis	Prevention of pig serum-induced rat liver fibrosis	Hironaka et al., 2000 [111]
Female BALB/c mice, Schistosome infection. GdCl <sub>3</sub> (10 mg/kg body weight), repeated administration.	GdCl <sub>3</sub> decreased macrophages infiltration in granulomas liver of mice infected with <i>S. japonicum</i> . Attenuated liver injury: smaller granuloma size and decreased immune inflammation as well as less fibrogenesis.	Zheng et al., 2015 [123]
Cholestasis induced by ANIT (alpha-naphthylisothiocyanate) in CBA/C57Bl/6 mice	Pretreatment with GdCl <sub>3</sub> increased the severity of intrahepatic cholestasis and signs of liver damage.	Korolenko et al., 2008 [113]
Intact Wistar rats	GdCl <sub>3</sub> increased uptake of labelled oxidized low-density lipoproteins by rat heart and aorta	Ussynin et al., 1999 [116]
Poloxamer 407-induced lipemia in CBA mice	GdCl <sub>3</sub> pre-treatment decreased serum lipids in acute lipemia during macrophage depression	Goncharova et al., 2016 [120]
C57BL/6 mice, GdCl <sub>3</sub> <sup>+</sup> atherogenic diet.	Reduction of liver inflammation and expression of inflammatory cytokines, reduced steatosis	Olteanu et al., 2014 [128]

Gadolinium chloride prevented death in rats after administration of non-sonicated zymosan particles, resulting in 70% survival, compared with 17% survival in rats administered zymosan alone. Thus, macrophage depression induced by  $\text{GdCl}_3$  pretreatment abolished symptoms of inflammation in this zymosan-model, possibly by influencing the cysteine proteinases of Kupffer cells [115]. Liver macrophage depression in mice induced by the administration of  $\text{GdCl}_3$  coincided with maximal accumulation of gadolinium in liver cells, as was shown by direct assay of gadolinium levels in hepatic tissue [112]. The kinetics of gadolinium accumulation was studied using inductively coupled plasma-emission spectroscopy after intravenous injection of  $\text{GdCl}_3$  (7.5-10 mg/kg) to CBA mice. Lysosomotropic  $\text{GdCl}_3$  was shown to exhibit long-term selective accumulation in liver lysosomes *in vivo*. Gadolinium uptake by hepatic cells attained a maximum 1 h after its intravenous injection and remained at this level for 24-48 h. Subsequently, the liver concentration of gadolinium was steadily declined for up to 30 days following the single dose. Accumulation of gadolinium in hepatocytic lysosomes disturbed their osmotic properties, as was determined from an increase in free acid phosphatase activity, and this effect persisted for 19 days [112, 113].

In previous experiments conducted by others, hepatoprotective effects of  $\text{GdCl}_3$  were observed in which it reversed dimethylnitrosamine (DMN)-induced rat liver fibrosis [117]. It was suggested that Kupffer cells can reverse liver fibrosis through the increased expression of MMPs, mainly through the P38 pathway. Hironaka et al. [111] clearly demonstrated that Kupffer cells are a major source of interstitial collagenase (MMP-13 or collagenase-3), which plays an important role in fibrolysis and consequently, prevents development of liver fibrosis. This evidence suggests a new therapeutic strategy for the treatment of liver fibrosis. Ide et al. [118] discovered that decreased numbers of macrophages may contribute to improvement of hepatic fibrosis in rats with thioacetamide-induced fibrosis. Positive effects of  $\text{GdCl}_3$  in cycloheximide-induced liver injury were attributed to increased secretion of anti-inflammatory cytokines [124]. Thus, liver macrophages can play a significant role in abolishing necroinflammatory changes in the liver of experimental animals in which the administration of cycloheximide would normally induce apoptosis.

$\text{GdCl}_3$  significantly reduces ED2 antigen expression by Kupffer cells *in vivo* [121]. ED2 expression of cultured Kupffer cells *in vitro* is not affected by  $\text{GdCl}_3$  (0-27 microM) [121]. Other researchers [119] have discovered that the combination of  $\text{GdCl}_3$  and Sorafenib (a tyrosine kinase inhibitor for anti-angiogenesis treatment) can suppress collagen accumulation in liver cells,

suggesting that such a new approach may be a potential therapeutic strategy in the treatment of liver fibrosis.

It has also been shown that administration of  $\text{GdCl}_3$  to intact CBA/Lac mice was followed by an increase in serum cholesterol concentrations during the period of liver macrophage depression (24 h), but not during the period of macrophage repopulation (i.e., at 3-7 days after  $\text{GdCl}_3$  administration) [120]. However, preliminary injection of  $\text{GdCl}_3$  to mice 24 h before the induction of acute lipemia using the hyperlipidemic agent (poloxamer 407), reduced the concentrations of triglycerides and LDL-cholesterol during the period in which there was marked depression of macrophages (24 h). Macrophage repopulation (3-7 days after  $\text{GdCl}_3$  administration) was associated with an increase in both serum triglycerides and LDL-cholesterol levels in contrast to the trends in lipid levels observed when  $\text{GdCl}_3$  was administered to non-hyperlipidemic CBA/Lac mice. Electron microscopic analysis of Kupffer cells after the injection of poloxamer 407 and its combination with  $\text{GdCl}_3$  resulted in an intralysosomal accumulation syndrome in liver cells, as well as the formation of auto- and hetero-phagolysosomes. The activity of cathepsin B (a biomarker cysteine protease activity in liver macrophages), was significantly reduced 24 h after injection of either  $\text{GdCl}_3$  or poloxamer 407 alone, but restored in experiments wherein both agents were administered together. Selective depression of liver macrophages at 24-48 h after administration of  $\text{GdCl}_3$  has been shown to be accompanied by a decrease in serum chitotriosidase activity. Thus, it has been concluded that the accumulation of gadolinium in the lysosomes of liver macrophages leads to their damage and the elimination of a specific population of macrophages (primarily large cells). Changes in the activity of serum lysosomal enzymes also reflects repopulation of macrophages in the liver.

Proliferation of hepatic macrophages and the subsequent production of pro-inflammatory cytokines induce inflammatory cascades, transcription factors involved in lipid metabolism/translocation, and modulates programmed cell death [125, 126]. These findings support the pathophysiological role of macrophages in the pathogenesis of liver steatosis. Evaluating potential therapeutic targets against the infiltration and/or polarization of specific macrophage subtypes could potentially be of clinical interest [125].

## CONCLUSION

In this chapter, we have discussed hyperlipidemia and its impact on the development of CVD. Next we presented the use of statins, as well as both yeast

polysaccharides and other miscellaneous newer and investigational non-statin agents with which to treat hyperlipidemia and consequently reduce the development of CVD. The very important processes of autophagy and especially lipophagy were discussed in view of lipid accumulation in the liver, which is responsible for various liver pathologies such as liver steatosis, steatohepatitis, and fibrotic liver. Lastly, we presented the gadolinium-induced model of macrophage depression in experimental biology and medicine. This model allows investigators to specifically evaluate the function or role of liver macrophages in various liver pathologies, as well as to intentionally modulate the population of liver macrophages that can potentially participate in lipid turnover and lipid homeostasis. This model allows for a deeper understanding of lipid storage syndromes that affect normal liver function and the precise way in which intralysosomal storage of lipids in macrophages occurs. This may perhaps give rise to new treatment paradigms for liver disease.

## REFERENCES

- [1] American Heart Association. Heart disease and stroke statistics - 2016 update. <http://circ.ahajournals.org/content/133/4/447>. Accessed August 9, 2016.
- [2] Istituto Nazionale di Statistica. *Annuario statistic Italiano*. 2002, ISTAT; 2002.
- [3] McGill, HC; McMahon, A; Herderick, EE; Malcom, GT; Tracy, RE; Strong, JP. For the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) Research Group. Origin of atherosclerosis in childhood and adolescence. *Am. J. Clin. Nutr.* 2000, 72:1307S-1315S.
- [4] Napoli, C; Glass, CK; Witztum, JL; Deutsch, R; D'Armiento, FP; Palinski, W. Influence of maternal hypercholesterolemia during pregnancy on progression of early atherosclerotic lesions in childhood: Fate of Early Lesions in Children (FELIC) study. *Lancet* 1999, 354:1234-1241.
- [5] Pathological Determinants of Atherosclerotic in Youth (PDAY) Research Group. Natural history of aortic and coronary atherosclerotic lesions in youth. Findings from the PDAY Study. *Arterioscler. Thromb.* 1993, 13:1291-1298.
- [6] Tracy, RE; Newman III, WP; Wattigney, WA. Histological features of atherosclerosis and hypertension from autopsies of young individuals in a

- defined geographic population: Bogalusa Heart Study. *Atherosclerosis* 1995, 116:163-179.
- [7] Berenson, GS; Srinivasan, S. Cholesterol as a risk factor for early atherosclerosis: the Bogalusa Heart Study. *Prog. Pediatr. Cardiol.* 2003, 17:113-122.
- [8] Last, AR; Ference, JD; Flaaeroni, JF. Pharmacological treatment of hyperlipidemia. *Am. Fam. Physician* 2011, 84(5): 551-558.
- [9] Sattar, N; Preiss, D; Murray, HM; Welsh, P; Buckley, BM; de Craen, AJ; Seshasai, SR; McMurray, JJ; Freeman, DJ; Jukema, JW; Macfarlane, PW; Packard, CJ; Stott, DJ; Westendorp, RG; Shepherd, J; Davis, BR; Pressel, SL; Marchioli, R; Marfisi, RM; Maggioni, AP; Tavazzi, L; Tognoni, G; Kjekshus, J; Pedersen, TR; Cook, TJ; Gotto, AM; Clearfield, MB; Downs, JR; Nakamura, H; Ohashi, Y; Mizuno, K; Ray, KK; Ford, I. Statins and risk of incident diabetes: a collaborative meta-analysis of randomized statin trials. *Lancet* 2010, 375(9716): 735-742.
- [10] Hu, M; Cheung, BMY; Tomlinson, B. Safety of statins: an update. *Ther. Adv. Drug Saf.* 2012, 3(3): 133-144.
- [11] Weng, TC; Yang, YH; Lin, SJ; Tai, SH. A systematic review and meta-analysis on the therapeutic equivalence of statins. *J. Clin. Pharm. Ther.* 2010, 35(2):139-151.
- [12] Grundy, SM; Cleeman, JI; Merz, CN; Brewer, HB Jr; Clark, LT; Hunninghake, DB; Pasternak, RC; Smith, SC Jr; Stone, SJ. National Heart, Lung, and Blood Institute; American College of Cardiology Foundation; American Heart Association. Implications of recent clinical trials for the National Cholesterol Education Program Adult Treatment Panel III guidelines. *Circulation* 2004, 110(2): 227-239. [published erratum appears in *Circulation* 2004, 110(6):763].
- [13] Brugts, JJ; Yetgin, T; Hoeks, SE; Gotto, AM; Shepherd, J; Westendorp, RG; de Craen AJ; Knopp, RH; Nakamura, H; Ridker, P; van Domburg, R; Deckers, JW. The benefits of statins in people without established cardiovascular disease but with cardiovascular risk factors: meta-analysis of randomized controlled trials. *BMJ* 2009, 338:b2376.
- [14] Ray, KK; Seshasai, SR; Erqou, S; Sever, P; Jukema, JW; Ford, I; Sattar, N. Statins and all-cause mortality in high-risk primary prevention: a meta-analysis of 11 randomized controlled trials involving 65,229 participants. *Arch. Intern. Med.* 2010, 170(12):1024-1031.
- [15] Sacks, FM; Pfeffer, MA; Moye, LA; Rouleau, JL; Rutherford, JD; Cole, TG; Brown, L; Warnica, JW; Arnold, JM; Wun, CC; Davis, BR; Braunwald, E. The effect of pravastatin on coronary events after

- myocardial infarction in patients with average cholesterol levels. Cholesterol and Recurrent Events Trial Investigators. *N. Engl. J. Med.* 1996, 335(14):1001-1009.
- [16] Lewis, SJ; Moye, LA; Sacks, FM; Johnstone, DE; Timmis, G.; Mitchell, J; Limacher, M; Kell, S; Glasser, SP; Grant, J; Davis, BR; Pfeffer, MA; Braunwald, E. Effect of pravastatin on cardiovascular events in older patients with myocardial infarction and cholesterol levels in the average range. Results of the Cholesterol and Recurrent Events (CARE) trial. *Ann. Intern. Med.* 1998, 129(9): 681-689.
- [17] Serruys, PW; de Feyter, P; Macaya, C; Kokott, N; Puel, J; Vrolix, M; Branzi, A; Bertolami, MC; Jackson, G; Strauss, B; Meier, B; Lescol Intervention Prevention Study (LIPS) Investigators. Fluvastatin for prevention of cardiac events following successful first percutaneous coronary intervention: a randomized controlled trial. *JAMA* 2002, 287(24): 3215-3222.
- [18] Malik, P; Berisha, SZ; Santore, J; Agatista-Boyle, C; Brubaker, G; Smith, JD. Zymosan-mediated inflammation impairs *in vivo* reverse cholesterol transport. *J. Lipid Res.* 2011, 52(5): 951-957. doi: 10.1194/jlr.M011122
- [19] Korolenko, TA; Tuzikov, FV; Cherkanova, MS; Johnston, TP; Tuzikova, NA; Loginova, VM; Filjushina, EE; Kaledin, VI. Influence of atorvastatin and carboxymethylated glucan on the serum lipoprotein profile and MMP activity of mice with lipemia induced by poloxamer 407. *Can. J. Physiol. Pharmacol.* 2012, 90(2):141-153. doi: 10.1139/y11-118.
- [20] Korolenko, TA; Kisarova, YA; Filjushina, EE; Dergunova, MA; Machova, E. Macrophage stimulation and  $\beta$ -D-glucans as biological response modifiers: the role in experimental tumor development. In R. Takahashi, H. Kai. (Eds.), *Handbook of Macrophages: Life Cycle, Functions and Diseases*, New York: Nova Science Publishing; 2012; 249-276.
- [21] Napolitano, M; Sennato, S; Botham KM; Bordi, F; Bravo, E. Role of macrophage activation in the lipid metabolism of postprandial triacylglycerol-rich lipoproteins. *Exp. Biol. Med.* (Maywood) 2013, 238:98.
- [22] Greaves, DR; Gordon, S. Thematic review series: the immune system and atherogenesis. Recent insights into the biology of macrophage scavenger receptors. *J. Lipid Res.* 2005, 46(1): 11-20.
- [23] Matthijsen, RA; de Winther, MP; Kuipers, D; van der Made, I; Weber, C; Herias, MV; Gijbels, MJ; Buurman, WA. Macrophage-specific expression of mannose-binding lectin controls atherosclerosis in low-



- density-lipoprotein receptor-deficient mice. *Circulation*. 2009, 119:2188-2195.
- [24] Nguyen, DG; Hildreth, JE. Involvement of macrophage mannose receptor in the binding and transmission of HIV by macrophages. *Eur. J. Immunol*. 2003, 33(2):483-493.
- [25] Korolenko, TA; Rukavishnikova, EV; Safina, AF; Dushkin, MI; Mynkina, GI. Endocytosis by liver cells during suppression of intralysosomal proteolysis. *Biol. Chem. Hoppe. Seyler*. 1992, 373(7): 573-80. PMID: 1515086.
- [26] Poteryaeva, ON; Falameyeva, OV; Korolenko, TA; Kaledin, VI; Djanayeva, SJ; Nowicky, JW; Sandula, J. Cysteine proteinase inhibitor level in tumor and normal tissues in control and cured micw. *Drugs Exp. Clin. Res*. 2000, 26(5-6): 301-306. PMID: 11345042.
- [27] Alipour, A; Elte, JWF; van Zaanen, HCT; A.P. Rietveld, AP; Castro-Cabezas, M. Novel aspects of postprandial lipemia in relation to atherosclerosis. *Atherosclerosis Supplements* 2008, 9:39-44.
- [28] van Eijk, M; van Roomen, CP; Renkema, GH; Bussink, AP; Andrews, L; Blommaart, EF; Sugar, A; Verhoeven, AJ; Boot, RG; Aerts, JM. Characterization of human phagocyte-derived chitotriosidase, a component of innate immunity. *Int. Immunol*. 2005, 17:1505-1512.
- [29] Kraemer, WJ; Vingren, JL; Silvestre, R; Spiering, BA; Hatfield, DL; Ho, JY; Fragala, MS; Maresh, CM; Volek, JS. Effect of adding exercise to a diet containing glucomannan. *Metabolism* 2007, 56:1149-1158.
- [30] Korolenko, TA; Pisareva, EE; Filyushina, EE; Johnston, TP; Machova, E. Serum cystatin C and chitotriosidase in acute P-407 induced dyslipidemia: Can they serve as potential early biomarkers for atherosclerosis? *Exp. Toxicol. Pathol*. 2015, 67(9):459-466. doi: 10.1016/j.etp.2015.06.003.
- [31] Pisareva, EE; Goncharova, IA; Tuzikov, FV; Goncharova, NV; Makhova, E; Korolenko, TA. Role of changes in serum chitotriosidase activity in mice under conditions of hyperlipidemia and lipid-lowering effect of carboxymethylated (1-3)- $\beta$ -D-glycan. *Bull. Exp. Biol. Med*. 2014, 157(5): 555-559.
- [32] Pollack, OJ. Reduction of blood cholesterol in man. *Circulation* 1953, 2:702-706.
- [33] Weststrate, JA; Meijer, GW. Plant sterol-enriched margarines and reduction of plasma total- and LDL-cholesterol concentrations in normocholesterolemic and mildly hypercholesterolemic subjects. *Eur. J. Clin. Nutr*. 1998, 52:334-343.

- [34] Hallikainen, MA; Sarkkinen, ES; Gylling, H; Erkkila, AT; Uusitupa, MIJ. Comparison of the effects of plant sterol ester and plant stanol ester-enriched margarines in lowering serum cholesterol concentrations in hypercholesterolaemic subjects on a low fat diet. *Eur. J. Clin. Nutr.* 2000, 54: 715-725.
- [35] Vanstone, CA; Raeini-Sarjaz, M; Parsons, WE; Jones, PJ. Unesterified plant sterols and stanols lower LDL-cholesterol concentrations equivalently in hypercholesterolemic persons. *Am. J. Clin. Nutr.* 2002, 76:1272-1278.
- [36] Varady, KA; Ebine, N; Vanstone, CA; Parsons, WE; Jones, PJ. Plant sterols and endurance training combine to favorably alter plasma lipid profiles in previously sedentary hypercholesterolemic adults after 8 wk. *Am. J. Clin. Nutr.* 2004, 80:1159-1166.
- [37] Ikeda, I; Tanaka, K; Sugano, M; Vahouny, GV; Gallo, LL. Inhibition of cholesterol absorption in rats by plant sterols. *J. Lipid Res.* 1988, 29:1573-1582.
- [38] Nissinen, M; Gylling, H; Vuoristo, M; Miettinen, TA. Micellar distribution of cholesterol and phytosterols after duodenal plant sterol ester infusion. *Am. J. Physiol. Gastrointest. Liver Physiol.* 2002, 282:G1009-G1015.
- [39] Doi, K; Masuura, M; Kawara, A; Baba, S. Treatment of diabetes with glucomannan. *Lancet* 1979, 1:987-988. (abstract).
- [40] Arvill, A; Bodin, L. Effect of short-term ingestion of glucomannan on serum cholesterol in healthy men. *Am. J. Clin. Nutr.* 1995, 61:585-589.
- [41] Vuksan, V; Sievenpiper, JL; Owen, R; Swilley, JA; Spadafora, P; Jenkins DJ; Vidgen, E; Brighenti, F; Josse, RG; Leiter, LA; Xu, Z; Novokmet, R. Beneficial effects of viscous dietary fiber from konjac-mannan in subjects with the insulin resistance syndrome. *Diabetes Care* 2000, 23:9-14.
- [42] Chen, HL; Shen, WH; Tai, TS; Liaw, YP; Chen, YC. Konjac supplement alleviated hypercholesterolemia and hyperglycemia in type 2 diabetic subjects - a randomized double-blinded trial. *J. Am. Coll. Nutr.* 2003, 22:36-42.
- [43] Doi, K; Nakamura, T; Aoyama, N; Matsuura, M; Kawara, A. Metabolic and nutritional effects of long-term use of glucomannan in the treatment of obese diabetics. In Y. Oomura, S. Tarui, S. Inoue, T. Shimazu (Eds.), *Progress in Obesity Research 1990. Proceedings of the Sixth International Congress on Obesity*, London: John Libbey; 1990; 507-514.
- [44] Huang, CY; Zhang, MY; Peng, SS; Hong, JR; Wang, X; Jiang, HJ; Zhang, FL; Bai, YX; Liang, JZ; Yu, YR; *et al.* Effect of konjac food on blood

- glucose level in patients with diabetes. *Biomed. Environ. Sci.* 1990, 3:123-131.
- [45] Doi, K. Effects of konjac fiber (glucomannan) on glucose and lipids. *Eur. J. Clin. Nutr.* 1995, 49:S190-S197.
- [46] Vuksan, V; Sievenpiper, JL; Xu, Z; Wong, EY; Jenkins, AL; Beljan-Zdravkovic, U; Leiter, LA; Josse, RG; Stavro, MP. Konjac-Mannan and American ginseng: emerging alternative therapies for type 2 diabetes mellitus. *J. Am. Coll. Nutr.* 2001, 20:370S-380S.
- [47] Jones, PJ; Leitch, CA; Pederson, RA. Meal-frequency effects on plasma hormone concentrations and cholesterol synthesis in human. *Am. J. Clin. Nutr.* 1993, 57:868-874.
- [48] Lakshmanan, MR; Nepokroeff, CM; Ness, GC; Dugan, RE; Porter, JW. Stimulation by insulin of rat liver 3-hydroxy-3-methylglutaryl coenzyme A reductase and cholesterol synthesizing activity. *Biochem. Biophys. Res. Commun.* 1973, 50:704-710.
- [49] Matsuura, M. Effects of dietary fiber (glucomannan) on serum cholesterol. *Jpn. Soc. Clin. Nutr.* 1986, 8:1-11.
- [50] Gallaher, CM; Munion, J; Hesslink Jr, R; Wise, J; Gallaher, DD. Cholesterol reduction by glucomannan and chitosan is mediated by changes in cholesterol absorption and bile acid and fat excretion in rats. *J. Nutr.* 2000, 130(11):2753-2759.
- [51] Livieri, C; Novanzi, F; Lorini, R. The use of highly-purified glucomannan-based fibers in childhood obesity. *Pediatr. Med. Chir.* 1992, 14:196-198.
- [52] Yoshida, M; Vanstone, CA; Parsons, WD; Zawistowski, J; Jones, PJH. Effect of plant sterols and glucomannan on lipids in individuals with and without type II diabetes. *Eur. J. Clin. Nutr.* 2006, 60(4):529-537
- [53] Martino, F; Martino, D; Morrone, F; Carnevali, E; Forcone, R; Niglio, T. Effect of dietary supplementation with glucomannan on plasma total cholesterol and low density lipoprotein cholesterol in hypercholesterolemic children. *Nutr. Metab. Cardiovasc. Dis.* 2005, 15:174-180.
- [54] Jenkins, DJA; Kendall, CWC; Vuksan, V. Viscous fibers, health claims, and strategies to reduce cardiovascular disease risk. *Am. J. Clin. Nutr.* 2000, 71:401-402.
- [55] Haskell, WL; Spiller, GA; Jensen, CD; Ellis, BK; Gates, JE. Role of water-soluble dietary fiber in the management of elevated plasma cholesterol in healthy subjects. *Am. J. Cardiol.* 1992, 69(5): 433-439.

- [56] Stein, EA. Other therapies for reducing low-density lipoprotein cholesterol: medications in development. *Endocrinol. Metab. Clin. North Am.* 2009, 38:99-119.
- [57] Toth, PP. Antisense therapy and emerging applications for the management of dyslipidemia. *J. Clin. Lipidol.* 2011, 5:441-449.
- [58] Kastelein, JJ; Wedel, MK; Baker, BF; Su, J; Bradley, JD; Yu, RZ; Chuang, E; Graham, MJ; Crooke, RM. Potent reduction of apolipoprotein B and low-density lipoprotein cholesterol by short-term administration of an antisense inhibitor of apolipoprotein. *Circulation* 2006, 114:1729-1735.
- [59] Cuchel, M; Bloedon, L; Szapary, P; Kolansky, DM; Wolfe, ML; Sarkis, A; Millar, JS; Ikewaki, K; Siegelman, ES; Gregg, RE; Rader, DJ. Inhibition of microsomal triglyceride transfer protein in familial hypercholesterolemia. *N. Engl. J. Med.* 2007, 356:148-156.
- [60] Patel, RS; Scopelliti, EM; Savelloni, J. Therapeutic management of familial hypercholesterolemia: current and emerging drug therapies. *Pharmacotherapy* 2015, 35(12): 1189-1203.
- [61] Aegerion Pharmaceuticals, Inc. Juxtapid (lomitapide) package insert. Cambridge, MA; 2015.
- [62] Yadav, K; Sharma, M; Ferdinand, KC. Proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors: present perspectives and future horizons. *Nutr. Metabol. Cardiovasc. Dis.* 2016, <http://dx.doi.org/10.1016/j.numecd.2016.05.006>.
- [63] Mullard, A. Cholesterol-lowering blockbuster candidates speed into Phase III trials. *Nat. Rev. Drug Disc.* 2012, 11:817-819.
- [64] Farnier, M. PCSK9 inhibitors. *Curr. Opin. Lipidol.* 2013, 24:251-258.
- [65] McDonagh, M; Peterson, K; Holzhammer, B; Fazio, S. A systematic review of PCSK9 inhibitors alirocumab and evolocumab. *J. Manag. Care Spec. Pharm.* 2016, 22(6):641-653.
- [66] Sanofi-Aventis U.S. LLC. Praluent (alirocumab) package insert. Bridgewater, NJ; 2015.
- [67] Amgen, Inc. Repatha (evolocumab) package insert. Thousand Oaks, CA; 2015.
- [68] McKenney, JM; Koren, MJ; Kereiakes, DJ; Hanotin, C; Ferrand, A; Stein, EA. Safety and efficacy of a monoclonal antibody to proprotein convertase subtilizing/kexin type 9 serine protease, SAR236553/REGN727, in patients with primary hypercholesterolemia receiving ongoing stable atorvastatin therapy. *J. Am. Coll. Cardiol.* 2012, 59:2344-2353.

- [69] Santos, RD. Review: PCSK9 inhibitors reduce mortality but increase neurocognitive events in hypercholesterolemia. *Ann. Intern. Med.* 2016, 164(6):JC31. doi 10.7326/ACPJC-2016-164-6-031.
- [70] Yamashita, S; Matsuzawa, Y. Re-evaluation of cholesteryl ester transfer protein function in atherosclerosis based upon genetics and pharmacological manipulation. *Curr. Opin. Lipidol.* 2016, 27(5):459-472 doi 10.1097/MOL.0000000000000332.
- [71] Ikewaki, K; Rader, DJ; Sakamoto, T; Nishiwaki, M; Wakimoto, N; Schaefer, JR; Ishikawa, T; Fairwell, T; Zech, LA; Nakamura, H; Nagano, M; Brewer Jr., HB. Delayed catabolism of high density lipoprotein apolipoproteins A-I and A-II in human cholesteryl ester transfer protein deficiency. *J. Clin. Invest.* 1993, 92:1650-1658.
- [72] Barter, PJ; Caulfield, M; Eriksson, M; Grundy, SM; Kastelein, JJ; Komajda, M; Lopez-Sendon, J; Mosca, L; Tardif, JC; Waters, DD; Shear, CL; Revkin, JH; Buhr, KA; Fisher, MR; Tall, AR; Brewer, B; ILLUMINATE Investigators. Effects of torcetrapib in patients at high risk for coronary events. *N. Engl. J. Med.* 2007, 357:2109-2122.
- [73] Sirtori, CR; Mombelli, G. Viability of developing CETP inhibitors. *Cardiovasc. Therapeut.* 2008, 26:135-146.
- [74] Miller, NE. CETP inhibitors and cardiovascular disease: time to think again. *F1000Res* 2014, 3:124. doi: 10.12688/f1000research.4396.1. eCollection 2014
- [75] Hovingh, GK; Ray, KK; Boekholdt, SM. Is cholesteryl ester transfer protein inhibition an effective strategy to reduce cardiovascular risk? CETP as a target to lower CVD risk: suspension of disbelief? *Circulation* 2015, 132:433-440.
- [76] Schaefer, EJ. Effects of cholesteryl ester transfer protein inhibitors on human lipoprotein metabolism: why have they failed in lowering coronary heart disease risk? *Curr. Opin. Lipidol.* 2013, 24:259-264.
- [77] Hendriks, T; Bieghe, V; Walenbergh, SM; van Gorp, PJ; Verheyen, F; Jeurissen, ML; Steinbusch, MM; Vaes, N; Binder, CJ; Koek, GH; Stienstra, R; Netea, MG; Hofker, MH; Shiri-Sverdlov, R. Macrophage specific caspase-1/11 deficiency protects against cholesterol crystallization and hepatic inflammation in hyperlipidemic mice. *PLoS One.* 2013, 8(12): e78792. doi: 10.1371/journal.pone.0078792. eCollection 2013.
- [78] Zhou, F; Liu, D; Ning, HF; Yu, XC; Guan, XR. The roles of p62/SQSTM1 on regulation of matrix metalloproteinase-9 gene expression in response

- to oxLDL in atherosclerosis. *Biochem. Biophys. Res. Commun.* 2016, 472(3): 451-458. doi: 10.1016/j.bbrc.2016.01.065.
- [79] Shen, L; Qi, Z; Zhu, Y; Song, X; Xuan, C; Ben, P; Lan, L; Luo, L; Yin, Z. Phosphorylated heat shock protein 27 promotes lipid clearance in hepatic cells through interacting with STAT3 and activating autophagy. *Cell. Signal.* 2016, 28: 1086–1098.
- [80] Wang, S; Pacher, P; De Lisle, RC; Huang, H; Ding, W.-X. A Mechanistic Review of Cell Death in Alcohol-Induced Liver Injury (Review) *Alcoholism: Clinical and Experimental Research.* 2016, 40 (6): 1215-1223.
- [81] Ding, W-X; Jaeschke, H. Autophagy in macrophages regulates the inflammasome and protects against liver injury. *J. Hepatol.* 2016, 64 (1): 16-18. doi: 10.1016/j.jhep.2015.10.003.
- [82] Su, P; Zhang, J; Wang, D; Zhao, F; Cao, Z; Aschner, M; Luo, W. The role of autophagy in modulation of neuroinflammation in microglia. *Neuroscience.* 2016, 319: 155–167.
- [83] Madrigal-Matute, J; Cuervo, AM. Regulation of Liver Metabolism by Autophagy. *Gastroenterology.* 2016, 150:328–339.
- [84] Ilyas, G; Zhao, E; Liu, K; Lin, Y; Tesfa, L; Tanaka, KE; Czaja, M.J. Macrophage autophagy limits acute toxic liver injury in mice through down regulation of interleukin-1 $\beta$ . *J. Hepatol.* 2016, 64 (1): 118-127.
- [85] Czaja, MJ. Function of Autophagy in Nonalcoholic Fatty Liver Disease. *Digestive Diseases and Sciences.* 2016, 61 (5): 1304-1313.
- [86] Luo, Y; Lu, S; Zhou, P; Ai, QD; Sun, GB; Sun, XB. Autophagy: An Exposing Therapeutic Target in Atherosclerosis. *J. Cardiovasc. Pharmacol.* 2016, 67(3): 266-274. doi: 10.1097/FJC.0000000000000342.
- [87] Martinez-Lopez, N; Singh, R. Autophagy and Lipid Droplets in the Liver. *Annu. Rev. Nutr.* 2015, 35:215-237. doi: 10.1146/annurev-nutr-071813-105336.
- [88] Kwanten, WJ; Martinet, W; Michielsen, PP; Francqu, S.M. Role of autophagy in the pathophysiology of nonalcoholic fatty liver disease: A controversial issue. *World J. Gastroenterol.* 2014; 20(23): 7325-7338.
- [89] Korolenko, TA; Cherkanova, MS; Tuzikov, FV; Johnston, TP; Tuzikova, NA; Loginova, VM; Kaledin, VI. Influence of atorvastatin on fractional and subfractional composition of serum lipoproteins and MMP activity in mice with Triton WR 1339-induced lipaemia. *J. Pharm. Pharmacol.* 2011, 63(6): 833-839. doi: 10.1111/j.2042-7158.2011.01287.x.
- [90] Korolenko TA, Tuzikov FV, Johnston TP, Tuzikova NA, Kisarova YA, Zhanaeva SY, Alexeenko TV, Zhukova NA, Brak IV, Spiridonov VK,

- Filjushina EE, Cherkanova MS, Monoszon AA. The influence of repeated administration of poloxamer 407 on serum lipoproteins and protease activity in mouse liver and heart. *Can. J. Physiol. Pharmacol.* 2012, 90(11):1456-1468. doi: 10.1139/y2012-118.
- [91] Liu, W; Baker, RD; Bhatia, T; Zhu, L; Baker, SS. Pathogenesis of nonalcoholic steatohepatitis. *Cell. Mol. Life Sci.* 2016, 73 (10): 1969-1987.
- [92] Cingolani, F; Czaja, MJ. Regulation and Functions of Autophagic Lipolysis. *Trends Endocrinol. Metab.* 2016 Jun 27. pii: S1043-2760(16)30064-9 doi: 10.1016/j.tem.2016.06.003.
- [93] Simon, TG; Bonilla, H; Yan, P; Chung, RT; Butt, AA. Atorvastatin and fluvastatin are associated with dose-dependent reductions in cirrhosis and hepatocellular carcinoma, among patients with hepatitis C virus: Results from ERCHIVES. *Hepatology.* 2016, 64(1): 47-57.
- [94] Yang, F; Wang, J; Li, F; Cui, L. Atorvastatin Combined Nitroglycerin Therapy Confer Additive Effects on Rabbits with Dyslipidemia. *Exp. Clin. Endocrinol. Diabetes.* 2016, 124(6): 367-371.
- [95] Salic, K; Morrison, MC; Verschuren, L; Wielinga, PY; Wu, L; Kleemann, R; Gjorstrup, P; Kooistra, T. Resolvin E1 attenuates atherosclerosis in absence of cholesterol-lowering effects and on top of atorvastatin. *Atherosclerosis.* 2016, 250:158-165. doi: 10.1016/j.atherosclerosis.2016.05.001.
- [96] Svechnikova, IG; Korolenko, TA; Stashko, JuF; Kaledin, VI; Nikolin, VP, Nowicky, JW. The influence of Ukrain on the growth of HA-1 tumor in mice: the role of cysteine proteinases as markers of tumor malignancy. *Drugs Exp. Clin. Res.* 1998, 24(5-6): 261-269.
- [97] Korolenko, TA; Svechnikova, IG; Filjushina, EE; Kaledin, VI; Vakulin, GM; Usynin, IF; Tsyrendordjiev, DD. Macrophage stimulation and antitumor effect of Ukrain. *Drugs Exp. Clin. Res.* 1998, 24(5-6): 253-260.
- [98] Glazer, HP; Robert, M; Osipov, RM; Clements, RT; Frank, W; Sellke, FW; Bianchi, C. Hypercholesterolemia is associated with hyperactive cardiac mTORC1 and mTORC2 signaling. *Cell Cycle.* 2009, 8(11): 1738-1746.
- [99] Orogo, AM; Gustafsson, AB. Therapeutic Targeting of Autophagy: Potential and Concerns in Treating Cardiovascular Disease. *Circ. Res.* 2015, 116(3): 489-503. doi:10.1161/CIRCRESAHA.116.303791
- [100] De Meyer, GR; Grootaert, MO; Michiels, CF; Kurdi, A; Schrijvers, DM; Martinet, W. Autophagy in vascular disease. *Circ Res.* 2015, 116(3): 468-479. doi: 10.1161/CIRCRESAHA.116.303804.

- [101] Grootaert, MO; da Costa Martins, PA; Bitsch, N; Pintelon, I; De Meyer, GR; Martinet, W; Schrijvers, DM. Defective autophagy in vascular smooth muscle cells accelerates senescence and promotes neointima formation and atherogenesis. *Autophagy*. 2015, 11(11): 2014-2032.
- [102] Li, L; Xu, J; He, L; Peng, L; Zhong, Q; Chen, L; Jiang, Z. The role of autophagy in cardiac hypertrophy. *Acta Biochim. Biophys. Sin. (Shanghai)*. 2016, 48(6): 491-500. doi: 10.1093/abbs/gmw025.
- [103] Dennemarker, J; Lohmuller, T; Muller, S; Aguilar, SV; Tobin, DJ; Peters, C; Reinheckel, T. Impaired turnover of autophagolysosomes in cathepsin L deficiency. *Biol. Chem*. 2010, 391: 913–922.
- [104] Sun, M; Ouzounian, M; de Couto, G; Chen, M; Yan, R; Fukuoka, M; Li, G; Moon, M; Liu, Y; Gramolini, A; Wells, GJ; Liu, PP. Cathepsin-L ameliorates cardiac hypertrophy through activation of the autophagy-lysosomal dependent protein processing pathways. *J. Am. Heart Assoc*. 2013, 2 (2): e000191. doi: 10.1161/JAHA.113.000191.
- [105] Dinger, SC; Fridjhon, P; Rubin, DM. Thermal Excitation of Gadolinium-Based Contrast Agents Using Spin Resonance. *PLoS One*. 2016 Jun 24; 11(6): e0158194. doi: 10.1371/journal.pone.0158194. eCollection 2016.
- [106] Kanal, E; Tweedle, MF. Residual or retained gadolinium: practical implications for radiologists and our patients. *Radiology*. 2015, 275:630–634.
- [107] Forghani, R. Adverse Effects of Gadolinium-Based Contrast Agents: Changes in Practice Patterns. *Top Magn. Reson. Imaging*. 2016, 25(4): 163-169. doi: 10.1097/RMR.0000000000000095.
- [108] Hardonk, MJ; Dijkhuis, FW; Hulstaert, CE; Koudstaal, J. Heterogeneity of rat liver and spleen macrophages in gadolinium chloride-induced elimination and repopulation. *J. Leukoc. Biol*. 1992; 52(3):296-302.
- [109] Golbar, HM; Yamate, J. Hepatic Macrophages and Macrophages with Different Functions in Hepatic fibrosis. In: *Handbook of Macrophages: Life Cycle, Functions and Diseases*. Editors: Rikiya Takahashi and Hibiki Kai. 2012. Nova Publishers, New York, USA (pp. 277-294).
- [110] Zimmermann, HW; Trautwein, C; Tacke, F. Functional role of monocytes and macrophages for the inflammatory response in acute liver injury. *Front. Physiol*. 2012. 19; 3:56. doi: 10.3389/fphys.2012.00056. eCollection 2012.
- [111] Hironaka, K; Sakaida, I; Matsumura, Y; Kaino, S; Miyamoto, K; Okita, K. Enhanced Interstitial Collagenase (Matrix Metalloproteinase-13) Production of Kupffer Cell by Gadolinium Chloride Prevents Pig Serum-Induced Rat Liver Fibrosis. *Biochem. Biophys. Res. Commun*. 2000,



- 267(1): 290-295. doi:10.1006/bbrc.1999.1910. <http://www.idealibrary.com>.
- [112] Korolenko, TA; Dergunova, MA; Alekseenko, TV; Zhanaeva, SY; Filyushina, EE; Filatova, TG. Intralysosomal accumulation of gadolinium and lysosomal damage during selective depression of liver macrophages in vivo. *Bull. Exp. Biol. Med.* 2006, 142(4): 391-394.
- [113] Korolenko, TA; Klishevich, MS; Cherkanova, MS; Alexeenko, TV; Zhanaeva, SY; Savchenko, NG; Goncharova, IA; Filjushina, EE. In vivo effect of selective macrophage suppression on the development of intrahepatic cholestasis in mice. *Bull. Exp. Biol. Med.* 2008; 146(4):396-400.
- [114] Ding, H; Peng, R; Reed, E; Li, QQ. Effects of Kupffer cell inhibition on liver function and hepatocellular activity in mice. *Int. J. Mol. Med.* 2003; 12(4): 549-557.
- [115] Korolenko, T; Svechnikova, I; Urazgaliyev, K; Vakulin, G; Djanaeva, S. Liver cysteine proteinases in macrophage depression induced by gadolinium chloride. *Advances in Experimental Medicine and Biology.* 1997, 421: 315-321.
- [116] Usynin, IF; Khar'kovsky, AV; Balitskaya, NI; Panin, LE. Gadolinium chloride-induced Kupffer cell blockade increases uptake of oxidized low-density lipoproteins by rat heart and aorta. *Biochemistry (Mosc).* 1999; 64(6): 620-624.
- [117] Sakaida, I; Hironaka, K; Terai, S; Okita, K. Gadolinium chloride reverses dimethylnitrosamine (DMN)-induced rat liver fibrosis with increased matrix metalloproteinases (MMPs) of Kupffer cells. *Life Sciences.* 2003, 72 (8), 943-959.
- [118] Ide, M; Kuwamura, M; Kotani, T; Sawamoto, O; Yamate, J. Effects of Gadolinium Chloride (GdCl<sub>3</sub>) on the Appearance of Macrophage Populations and Fibrogenesis in Thioacetamide-Induced Rat Hepatic Lesions. *J. Comp. Path.* 2005, 133: 92-102
- [119] Liu, C; Yang, Z; Wang, L; Lu, Y; Tang, B; Miao, H; Xu, Q; Chen, X. Combination of sorafenib and gadolinium chloride (GdCl<sub>3</sub>) attenuates dimethylnitrosamine(DMN)-induced liver fibrosis in rats. *BMC Gastroenterology.* 2015 Nov 16; 15:159. doi: 10.1186/s12876-015-0380-52015.
- [120] Goncharova, NV; Pupyshev, AB; Filyushina, EE; Loktev, KV; Korolenko, ETs; Lushnikova, EL; Molodykh, OP; Korolenko, TA; Churin, BV. Depression of Macrophages Modifies Serum Lipid Profile in

- Hyperlipidemia. *Bull. Exp. Biol. Med.* 2016, 160(5): 617-621. doi: 10.1007/s10517-016-3231-7.
- [121] Lee, CM; Yeoh, GC; Olynyk, JK. Differential effects of gadolinium chloride on Kupffer cells *in vivo* and *in vitro*. *Int. J. Biochem. Cell Biol.* 2004; 36(3):481-488.
- [122] de Duve, C; de Barsy, T; Poole, B; Trouet, A; Tulkens, P; Van Hoof, F. Commentary. Lysosomotropic agents. *Biochem. Pharmacol.* 1974; 23(18): 2495-2531.
- [123] Zheng, S; Lu, Q; Xu, Y; Wang, X; Shen, J; Wang, W. GdCl<sub>3</sub> Attenuates Schistosomiasis japonicum Egg-Induced Granulomatosis Accompanied by Decreased Macrophage Infiltration in Murine Liver. *PLoS One*, 2015; 10(8): e0132222. doi:10.1371/journal.pone.0132222.
- [124] Kumagai, K; Kiyosawa, N; Ito, K; Yamoto, T; Teranishi, M; Nakayama, H; Manabe, S. Influence of Kupffer cell inactivation on cycloheximide-induced hepatic injury. *Toxicology*. 2007, 241(3): 106-118.
- [125] Wu, R; Nakatsu, G; Zhang, X; Yu, J. Pathophysiological mechanisms and therapeutic potentials of macrophages in non-alcoholic steatohepatitis. *Expert Opinion on Therapeutic Targets*. 2016; 20(5): 615-26. doi: 10.1517/14728222.2016.1125883.
- [126] Nati, M; Haddad, D; Birkenfeld, AL; Koch, CA; Chavakis, T; Chatzigeorgiou, A. The role of immune cells in metabolism-related liver inflammation and development of non-alcoholic steatohepatitis (NASH). *Rev. Endocr. Metab. Disord.* 2016; 17(1): 29-39. doi: 10.1007/s11154-016-9339-2.
- [127] Radi, ZA; Koza-Taylor, PH; Bell, RR; Obert, LA; Runnels, HA; Beebe, JS; Lawton, MP; Sadis, S. Increased serum enzyme levels associated with Kupffer cell reduction with no signs of hepatic or skeletal muscle injury. *Am. J. Pathol.* 2011, 179(1): 240-247. doi: 10.1016/j.ajpath.2011.03.029.
- [128] Olteanu, S; Kandel-Kfir, M; Shaish, A; Almog, T; Shemesh, S; Barshack, I; Apte, RN; Harats, D; Kamari, Y. Lack of interleukin-1 $\alpha$  in Kupffer cells attenuates liver inflammation and expression of inflammatory cytokines in hypercholesterolaemic mice. *Dig. Liver Dis.* 2014, 46(5): 433-439. doi: 10.1016/j.dld.2014.01.156.

Partially supported (TAK) by grant N 16-04-01423 of the Russian Fund for Basic Research



### *Chapter 3*

## **PROTECTION AGAINST MITOCHONDRIAL AND CELL DAMAGE: A NEW APPROACH TO TREAT SEVERE HEMORRHAGIC SHOCK**

***Z. H. Zeng<sup>1</sup>, S. Q. Xu<sup>2</sup> and K. S. Zhao<sup>2,\*</sup>***

<sup>1</sup>Department of Critical Care Medicine, Nanfang Hospital, Southern  
Medical University, Guangzhou, China

<sup>2</sup>Department of Pathophysiology, Southern Medical University,  
Guangzhou, China

### **ABSTRACT**

Severe shock is a life-threatening situation with ineffective of anti-shock treatments. Therefore, the mechanism of severe shock should be studied in detail to find new approaches to treatment. In severe shock, mitochondrial damage is a common phenomenon among diverse organs, and administration of mitochondrial protectors might represent a new approach for shock therapy. According to the pharmacological effect, drugs targeting mitochondria in severe shock include following aspects: (1) inhibiting mitochondrial permeability transition pore opening; (2) attenuating reactive oxygen species production; (3) modulating inner ion ( $\text{Ca}^{2+}$ ,  $\text{K}^{+}$ ) channels; (4) ameliorating energy substrate metabolism; and (5) activating sirtuin 1/3. Some of these new therapeutic methods have been

---

\* Corresponding Author: kszhao@ymail.com.

confirmed in animal studies, which provided a potential clinical application in shock treatment.

Abbreviations: adenine nucleotide translocase, ANT; acridine orange, AO; arteriolar smooth muscle cells, ASMC; adenosine triphosphate, ATP; atractyloside, ATR; cyclic adenosine monophosphate, cAMP; cyclosporine A, CsA; catalase, CAT; cyclophilin D, CyPD; electron-transferring flavin proteins, ETFs; electron transport system, ETS; functional capillaries density, FCD; glutathione peroxidase, GSH-Px; mitochondrial dysfunction, MD; mitochondrial inner membrane, MIM; mitochondrial membrane potential,  $\Delta\Psi_m$ ; mitochondrial outside membrane, MOM; mitochondrial permeability transition pore, mPTP; N-acetylcysteine, NAC; phenyl-tert-butyl nitron, PBN; polydatin, PD; resveratrol, RSV; reactive oxygen species, ROS; superoxide dismutase, SOD; triphenylphosphonium, TPP<sup>+</sup>; voltage-dependent anion channel, VDAC.

## INTRODUCTION

Hemorrhage is a medical emergency that is frequently encountered by physicians in emergency rooms, operating rooms and intensive care units [1]. Severe hemorrhage is main reason for hemorrhagic shock. Shock is one of the main causes of death and disability worldwide. In the severe shock period, it might be difficult to save the patient's life even after treatment by blood transfusion and other anti-shock methods [2].

Until recently, the concept of tissue hypoxia was the central paradigm of organ dysfunction induced by shock and other diseases, which results from the failure to deliver oxygen to meet the organ's needs. Recently, this tissue hypoxia paradigm has been challenged [3]. It has been proposed that impaired cellular oxygen utilization, termed "cytopathic hypoxia", rather than inadequate oxygen delivery, might play a more important role in the development of hemorrhagic shock. The key issue is that, although oxygen therapy may deliver adequate oxygenation of hemoglobin and tissue tensions, the oxygen might not be available at the mitochondrial level. Mitochondria are the main producers of energy, in the form of adenosine triphosphate (ATP) production. Mitochondria are the primary consumers of cellular oxygen; therefore, increasing attention been paid to the role of mitochondrial function and dysfunction in the establishment of cytopathic hypoxia during hemorrhagic shock [4].

As a research hotspot in recent years, mitochondrial function is associated with the genesis of many diseases, such as cardiovascular diseases, diabetes mellitus, Alzheimer disease, Parkinson disease, and nonalcoholic steatohepatitis

[5]. Mitochondrial dysfunction (MD) often occurs in relation to the opening of the mitochondrial permeability transition pore (mPTP) with intracellular low ATP content in severe shock. Low intracellular ATP content might lead to the dysfunction of vital organs, which is difficult to treat during severe shock. Therefore, protection against mitochondrial damage is a novel approach to treat severe shock. This article summarizes the concept, pathogenesis, variables and treatment of MD in severe hemorrhagic shock.

## **1. THE CONCEPT OF MITOCHONDRIAL DYSFUNCTION**

In 1962 Luft reported the first case of mitochondrial respiratory chain deficiency [6], and since then, the concept of MD has been used widely in the field of cell biology; however, no precise definition of MD has been made. The main function of mitochondria is ATP production via oxidative phosphorylation. In addition, mitochondria are also the main resource of reactive oxygen species (ROS) generation and scavenging, which are involved in cell apoptosis; calcium regulation in the cytosol and mitochondrial matrix; decomposition and synthesis of metabolites; cell proliferation; cell metabolism; and transported of subcellular organelles to correct position in various cells. Generally speaking, abnormalities in any one of these functions may cause MD; however, MD mainly refers to dysfunction in mitochondrial energy generation.

## **2. THE PATHOGENESIS OF MD IN SEVERE SHOCK**

### **2.1. Opening of the mPTP**

To explore the possible pathogenesis of MD, we first introduce the vital role of mitochondrial permeability transition pore (mPTP). The mPTP is a non-selective, large-conductance channel that is closed under physiological conditions [7]. A classic model of the mPTP consists of a dimer of a voltage-dependent anion channel (VDAC) in the mitochondrial outside membrane (MOM) and a dimer of the adenine nucleotide translocase (ANT) in the mitochondrial inner membrane (MIM), forming a channel [7]. However, this model is no longer widely supported because genetic ablation of ANT or VDAC did not eliminate mPTP opening, which was detected by mitochondrial swelling and by changes in the mitochondrial membrane potential ( $\Delta\Psi_m$ ) [8, 9].

However, the roles of ANT and VDAC as regulatory subunits of the mPTP, together with a matrix protein, cyclophilin D (CyPD), have been indicated by several lines of evidence, as discussed below. In addition, a recent study by Leunget et al. [10] suggested that the mitochondrial phosphate carrier [inorganic phosphate carrier (PiC)] is also a major subunit of the mPTP, possibly forming a channel in the MIM. A putative structure of the mPTP is a complex that crosses the mitochondrial membrane, consisting of VDAC in the MOM and the ANT–PiC complex in the MIM [7] (Figure 1). Under physiological conditions, the mPTP shows low conductance and is closed; however, in the pathological state, such as hypoxia, the mPTP is open and shows high conductance, which is one of the main mechanisms that lead to MD.

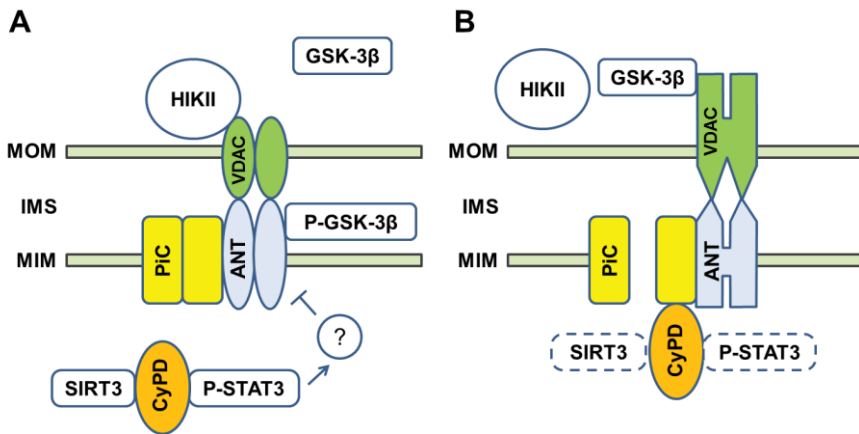


Figure 1. A model of the mitochondrial permeability transition pore (mPTP) interacting with regulatory proteins. (A) Closed mPTP and its interaction with inhibitory factors (HKII, p-GSK-3β, SIRT3, and p-STAT3); and (B) open mPTP and its sensitization to  $\text{Ca}^{2+}$  by binding of CyPD to PiC and by dissociation of HKII via VDAC phosphorylation by GSK-3β. Conformational change in the mPTP is induced by dissociation of HKII from VDAC and/or by binding of CyPD to PiC. HKII, hexokinase II; p-GSK-3β, glycogen synthase kinase-3β; STAT3, signal transducer and activator of transcription 3; VDAC, voltage-dependent anion channel; PiC, inorganic phosphate carrier; ANT, adenine nucleotide translocase; CyPD, cyclophilin D; MOM, mitochondrial outer membrane; IMS, intermembrane space; MIM, mitochondrial inner membrane.

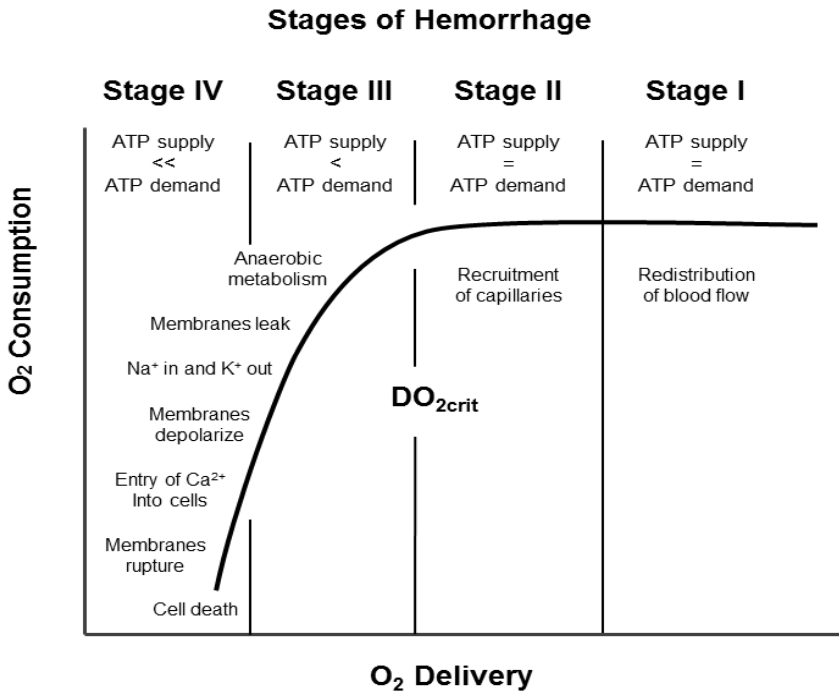


Figure 2. Changes in oxygen consumption shown as a function of oxygen delivery. Also shown are the hypothetical relationships of these parameters to the stages of hemorrhage and changes in cellular membrane integrity.  $DO_{2crit}$ , critical oxygen delivery.

## 2.2. Ischemia/Hypoxia and Microcirculation Dysfunction

Lower organ perfusion might be caused by a decrease in circulating blood volume during hemorrhage and depressed cardiac output. Severe hemorrhage impairs the delivery of oxygen and nutrients to the tissues, and produces a state of shock. However, little change in oxygen consumption is detected in the initial stage of shock, because blood flow is preferentially distributed to tissues with greater metabolic requirements [1]. Increased efficiency of oxygen utilization during hypoxia is reflected by a rise in the oxygen extraction ratio [11]. In spite of this organ-specific microvascular response, all organs, with the possible exception of the heart, experience decreases in blood flow during severe hypovolemia [12]. Another targeted response to hemorrhage is a decrease of the functional capillaries density (FCD) in organs. During severe hemorrhage stage,



the FCD decreases in proportion to the degree of tissue hypoxia [13], especially during the critical oxygen delivery period [1]. Severe and sustained decreases in oxygen delivery eventually overwhelm the microvascular responses to hypoxia. As tissue oxygen flux falters, mitochondria cannot sustain aerobic metabolism and oxygen consumption decreases [13].

In severe shock, energy depletion can induce the mPTP to open, and large amounts of lactic acid are generated in mitochondria via anaerobic oxidation. As electrons move down the proteins in the electron transport chain, the electrons lose energy to bring  $H^+$  ions from the mitochondrial matrix into the intermembrane space. The  $H^+$  attack the mPTP and induce its opening. Moreover, the antioxidant capacity of mitochondria decreases and many oxygen free radicals are produced, which also target the opening of the mPTP [14, 15].

### 2.3. Oxidative Stress

Oxidative stress is itself also intimately linked to MD. Mitochondria are generators of reactive oxygen species (ROS) and targets of ROS-mediated damage. As a major source of intracellular ROS, mitochondria produce ROS as a result of leakage of unpaired electrons, which are released from complexes I and III and their subsequent reaction with oxygen [16]. It is estimated that under normal conditions, 2–4% of electrons leak from the respiratory system; however, in disease states, this increases markedly, causing oxidative stress and cellular damage. In the healthy state, the mitochondrial defenses against oxidative stress, composed of antioxidants and ROS scavengers, including glutathione, manganese superoxide dismutase (MnSOD, also named SOD2), and the peroxiredoxins [17, 18], protect against widespread damage in the face of normal basal ROS production [19]. Once the balance of oxidation and anti-oxidation is disrupted, large amounts of synthesized oxygen free radical attack the membrane, nucleic acids and proteins of mitochondria, resulting in mitochondrial damage and mPTP opening, accelerating the production of more oxygen free radicals. Free radicals have deleterious effects on the function and integrity of mitochondria and further disturb cellular oxidative ATP production. Dysfunction of the electron transport system then generates more ROS and establishes a vicious circle. Disease states such as ischemia/reperfusion injury and shock, in which oxidative stress is a well-recognized phenomenon, are likely to be associated with concomitant MD [4]. Such dysfunction has deleterious effects on cellular oxygen utilization, energy generation, immune signaling and cell apoptosis. This may in turn initiate or augment subsequent

organ dysfunction and damage [4]. Notably, the anti-oxidative enzyme SOD2 plays a considerable role in scavenging overproduced ROS in mitochondria. Indeed, our research confirmed that both the protein content and the activity of SOD2 decreased sharply in the small intestine tissue [20, 21] and in hepatocytes [22] following hemorrhagic shock.

## 2.4. Calcium Overload

Free  $\text{Ca}^{2+}$  is a key signaling agent in eukaryotic cells. Hormonal and other stimuli activate phospholipase C, inositol triphosphate formation and mobilization of  $\text{Ca}^{2+}$  from the endoplasmic reticulum [23, 24]. In excitable tissues, membrane depolarization also causes  $\text{Ca}^{2+}$  entry through voltage gated  $\text{Ca}^{2+}$  channels. Increased cytosolic free  $\text{Ca}^{2+}$  then activates protein kinases, and in striated muscle, stimulates release of  $\text{Ca}^{2+}$  from sarcoplasmic reticulum to activate muscle contraction. Influx of  $\text{Ca}^{2+}$  into the cytosol also leads to mitochondrial accumulation of  $\text{Ca}^{2+}$  and activation of respiratory dehydrogenases and oxidative phosphorylation [25]. Most unstimulated cells tightly regulate their free  $\text{Ca}^{2+}$  in the range of 100 to 200 nM in the cytosol and the mitochondria through the action of membrane  $\text{Ca}^{2+}$  pumps in the plasma membrane and endoplasmic/sarcoplasmic reticulum; Na/Ca exchange across the plasma membrane; and, to a lesser extent, mitochondrial  $\text{Ca}^{2+}$  uptake. Thus, cytosolic free  $\text{Ca}^{2+}$  is substantially lower than the 2 mM free  $\text{Ca}^{2+}$  present in extracellular fluids [26]. After physiological stimulation, however, free  $\text{Ca}^{2+}$  in the cytosol and mitochondria can rapidly and transiently increase by 10 to 20-fold, as occurs on a beat-to-beat basis in cardiac myocytes because of mitochondrial  $\text{Ca}^{2+}$  uptake [27]. Dysregulation of  $\text{Ca}^{2+}$  homeostasis has long been thought to play an important role in cell injury. Pathological  $\text{Ca}^{2+}$  overload and calcification are frequently features of tissue ischemia and infarction, and increased  $\text{Ca}^{2+}$  activates a number of phosphatases, proteases and nucleases. Early experimental studies in hepatocytes showed that removal of extracellular  $\text{Ca}^{2+}$  protects against various hepatotoxicants, suggesting that the influx of extracellular  $\text{Ca}^{2+}$  is responsible for irreversible cell injury [28]. Maintenance of  $\text{Ca}^{2+}$  gradients across the plasma membrane and between cellular compartments depends on ATP-driven reactions; therefore, metabolic disruption by injurious stresses may perturb cellular  $\text{Ca}^{2+}$  homeostasis quickly. In particular, release of  $\text{Ca}^{2+}$  from the endoplasmic reticulum might flood the cytosol with free  $\text{Ca}^{2+}$ , possibly leading to activation of degradative processes and dysfunction of other organelles, particularly mitochondria [29]. Thus, once certain harmful factors destroy the homeostasis of mitochondria, abnormal calcium distribution or

calcium overload might occur [30]. The mitochondrial  $K_{ATP}$  inhibitor, 5-hydroxy decanoic acid salt, promote mitochondrial calcium overload and induces mPTP opening via inhibition of  $K^+$  uptake; mitochondrial ATP synthesis is then inhibited and the apoptotic process is initiated [31, 32].

## **2.5. Apoptosis Inducing Factors**

Evidence suggests that signals from healthy mitochondria can activate stress responses in cells, can activate apoptosis inducing factors, and can initiate suppression of metabolic activity mediated by activation of adenosine monophosphate (AMP)-dependent protein kinase (AMPK) [33]. Apoptosis inducing factors can induce mPTP opening and mitochondrial damage. These factors include: (1) endogenous factors: apoptotic pathway related protein Bax, Caspase-3, and caspase-9 [34]; and (2) exogenous factors: such as atractyloside (ATR) [35]. We demonstrated previously that the expressions of pro-apoptosis proteins p53, Bax and mitochondrial cytochrome C were increased in rat kidney tissue following hemorrhagic shock. Moreover, the MD was observed in renal tubular epithelial cells, as well as increased caspase-3 activity [34].

## **3. ASSESSING MD**

To explore mitochondrial function, certain methods are necessary. MD can be assessed in isolated mitochondria, in cells or in vivo, with different balances between precise experimental control and physiological relevance. Generally, measurements of fluxes give more information about the ability to make ATP than do measurements of intermediates or potentials. For isolated mitochondria, the best assay is mitochondrial respiratory control; i.e., the increase in respiration rate in response to ADP. However, the study of mitochondrial function in intact cells has greater physiological relevance. Moreover, the interactions with the rest of the cell are preserved in intact cell. Thus, in this chapter, we introduce mainly methods to measure MD in intact cells [36, 37].

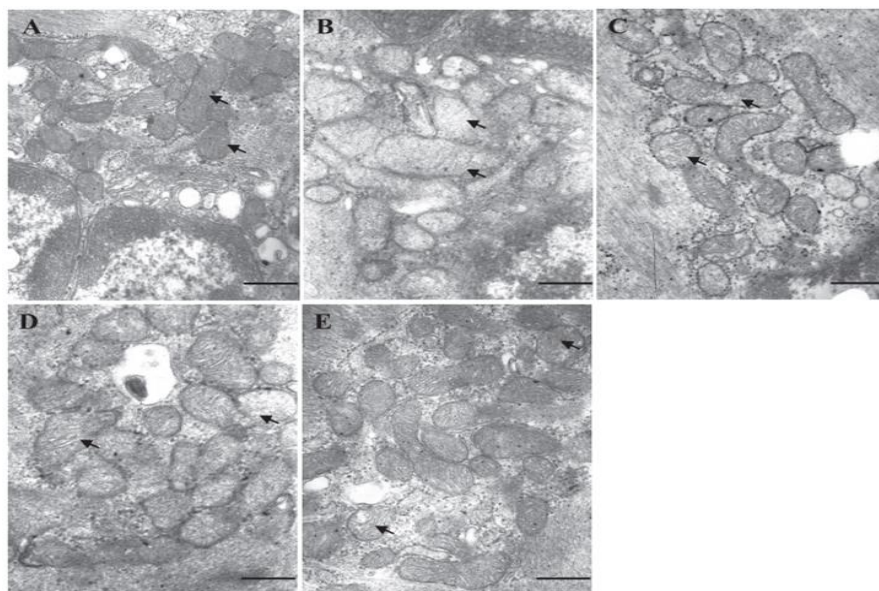


Figure 3. Ultrastructural alterations of arteriolar smooth muscle cells (ASMC) mitochondria following shock. Mitochondria are normal (some arrowed) in the control (sham) group (A). Mitochondria (some arrowed) are swollen with poorly defined cristae in the shock group (B), these alterations are partially prevented in the shock+CsA (C), shock + RSV (D), and shock + PD groups (E). CsA, Cyclosporine A; RSV, Resveratrol; PD, Polydatin.

### 3.1. Mitochondrial Morphology

Mitochondrial swelling is a prominent phenomenon in MD, which can be assessed semi-quantitatively using a scoring system based on the swelling level. Disruption of mitochondrial membranes and a loss of defined cristae accompany the increase in swelling. Mitochondrial swelling can occur through two distinct mechanisms: energy-dependent swelling and colloid osmotic swelling. Energy-dependent swelling is a consequence of solute accumulation (monovalent cations and anions), which is driven by the proton motive force or by simple concentration gradients. This kind of swelling appears without a loss of respiratory function. It is reversible and is not associated with “membrane damage”. Colloid osmotic swelling occurs when the mPTP opens, which is driven by the colloid osmotic pressure differential that exists across the inner mitochondrial membrane because proteins remain trapped within the matrix

space. Swelling of this kind can be difficult to reverse, and is accompanied by a loss of mitochondrial function [36, 38].

In pathological conditions, small molecular substances (molecular mass <1.5 kilodalton, such as  $H^+$  and metabolites) can cross the opened mPTP freely, elevating the mitochondrial colloid osmotic pressure, which increases the liquid content of the mitochondria, leading to swelling and structural damage [39]. Therefore, large amounts of swelling, disrupted mitochondrial membranes, and loss of defined cristae are important morphological signs of MD, which can be observed under a transmission electron microscope [40, 41].

Previously, we tested mitochondrial morphology in ASMCs following HS. Mitochondria in the control group appeared elliptical in shape with well-developed cristae and electron-dense matrices. In contrast, following HS, the mitochondria appeared irregularly shaped, swollen and disrupted, with poorly defined cristae and electron-lucent matrixes. These alterations were significantly restored by treatment with the mitochondrial protectors polydatin (PD) and resveratrol (RSV), respectively (Figure 3) [22] (The effects of both PD and RSV are discussed in section 4.6 in detail).

### 3.2. The Opening of mPTP

The mPTP is a multi-component protein aggregate in mitochondria that comprises proteins in the inner and outer membranes located at contact sites. The mPTP of the inner mitochondrial membranes normally remain closed, but can open under certain pathological condition, such as ischemia–reperfusion injury. Ischemia–reperfusion injury is associated with increase in mPTP activators ( $Ca^{2+}$ , ROS, Pi) and reductions in mPTP inhibitors (ATP/ADP). The open state of the mPTP comprises a channel  $\sim 3$ nm in diameter, thus allowing diffusion of all molecules with molecular masses less than 1.5 kDa. This causes equilibration of  $H^+$  across the inner membrane, which dissipates the  $\Delta\psi_m$ , leading to inhibition of ATP synthesis by the F1 F0-ATPase. A concomitant influx of water causes swelling of the mitochondria, which stretches the membranes to the point where the outer membrane fails. The permeabilization of the outer membrane to an extent allows the release of pro-apoptotic proteins (such as cytochrome C) from the inter-membrane space into the cytosol, resulting in apoptosis [42–44]. Therefore, the mPTP is a critical determinant for the genesis of MD and apoptosis. The assessment of mPTP opening can be measured by the  $^3H$ -DOG entrapment technique [45]. However, recently, the Calcein-AM and CoCl<sub>2</sub> techniques have replaced the DOG technique [46, 47], because  $^3H$ -DOG is a radioactive molecule. Calcein is a fluorescent molecule,

and upon its esterification to generate calcein-AM, it turns non-fluorescent. Once inside the cell, the probe is deesterified and trapped in its free, fluorophore form.  $\text{Co}^{2+}$  causes quenching of cytosolic and nuclear calcein, but it does not easily permeate the mitochondrial inner membrane. However, if the pores open,  $\text{Co}^{2+}$  can distribute itself inside mitochondria and result in quenched mitochondrial fluorescence. The density of mitochondrial fluorescence can be analyzed by flow cytometry [20, 34, 47].

### 3.3. Mitochondrial Membrane Potential

A reduced mitochondrial membrane potential ( $\Delta\psi\text{m}$ ) is another index to assess MD. A decrease in  $\Delta\psi\text{m}$  can be caused by two events. One is deficiency of oxidation or blockage of respiration, especially in hypoxia and shock, which leads to a reduction in  $\text{H}^+$  pumping into the intermembrane space. The other event is mPTP opening, which leads the  $\text{H}^+$  from the intermembrane space, where there is a high proton gradient, through the opened mPTP into the matrix, resulting in a low  $\Delta\psi\text{m}$ . ATP synthase is a reversible coupling enzyme that interconverts the energies of the electrochemical proton gradient and chemical bonds. A lower  $\Delta\psi\text{m}$  causes uncoupling of oxidative phosphorylation, which in turn causes the reverse mode activation of APTase leading to ATP hydrolysis rather than ATP synthesis. Mitochondrial membrane potential can be measured by fluorescent probes, such as JC-1, rhodamine 123 and TMRM. JC-1 monomers in the mitochondrial matrix (the presence of which indicates a low  $\Delta\psi\text{m}$ ) emit at 527 nm (green fluorescence), and JC-1 mitochondrial aggregates (indicating a normal  $\Delta\psi\text{m}$ ) emit at 590 nm (red fluorescence). A shift in fluorescence emission from red to green indicates depolarization. The percentage of cells with abnormally low  $\Delta\psi\text{m}$  (green fluorescence) can be measured by flow cytometry and immunofluorescence assays [48, 49].

### 3.4. Cellular ATP Content

Production of ATP is the main function of mitochondria. A reduced intracellular ATP content is an important variable to determine MD [50]. However, a decrease in ATP may also come from the insufficient delivery of oxygen and nutrient during shock. Therefore, the assessment of MD should combine the intracellular ATP level with other pathological, morphological and metabolic indices. The ATP level of isolated cells is usually measured by a

luciferase-based assay and the luminescence is tested using an automatic microplate reader [2, 38].

### **3.5. Lysosomal Membrane Permeabilization and Intracellular Lipid Peroxides Content**

Lysosome rupture with release of chelatable ferrous iron, ROS and cathepsins, has been demonstrated to induce mitochondrial damage including mPTP opening. Ischemia–reperfusion induces severe oxidative stress that leads to iron-mediated intra lysosomal production of hydroxyl radicals (Fento-type reactions) with ensuing lysosomal membrane permeabilization (LMP). Under conditions of LMP, relocated lysosomal enzymes may attack mitochondria through the so-called “Lysosomal-Mitochondrial Axis” [51, 52]. Therefore, measuring LMP and the intracellular lipid peroxides (LPO) level are indirect variables to assess MD. Lysosomal membrane stability is usually measured using an acridine orange (AO) uptake test. AO is a classic lysosomotropic fluorochrome, exhibiting red florescence when highly concentrated (e.g., in intact lysosomes). The percentage of “Pale” cells (i.e., cells with fewer than normal intact, red lysosomes) may be used to identify lysosomal membrane permeabilization [47].

### **3.6. Efficiency of Mitochondrial Protectors**

Treatment with mitochondrial protectors can attenuate the reduced intracellular ATP content with other pathological variables mentioned above during severe shock, which leads to the reduction of  $K_{ATP}$  channel activation and arterial smooth muscle cells hyperpolarization. The effects of mitochondrial protectors present indirect evidences of MD in vivo during severe shock. Therefore, the efficiency of a mitochondrial protector also is an assessment index of MD. According to the mechanism, the mitochondrial protectors can be divided into different groups [53–55]. One group inhibits mPTP opening, such as cyclosporine A (CsA), which is assumed to bind to CyP-D with nanomolar affinity and prevents the interaction of this protein with the adenine nucleotide translocator, thus inhibiting mPTP opening. The second group inhibits ROS generation in mitochondria, such as trans-resveratrol (RSV), which can decrease complex III activation with less ROS generation. PD is a glucoside of RSV, a natural polyphenolic compound [20, 56]. Both PD and RSV are stilbene-type of

compounds and share the same pharmacological effects (see section 4.6). The third group inhibits inner membrane  $\text{Ca}^{2+}$  regulated channels, such as ruthenium red, which can chelate  $\text{Ca}^{2+}$  or inhibit mitochondrial uniporters, preventing mPTP opening [27]. The fourth group activates MitoK<sub>ATP</sub> channels, such as diazoxide, which shows a protective effect against ischemia-reperfusion injury in the heart and neurons; however, the exact mechanism remains unclear. The beneficial effect of diazoxide could be the consequence of the partial dissipation of the membrane potential caused by the net influx of  $\text{K}^+$ . This would reduce  $\text{Ca}^{2+}$  entry or release an excess of  $\text{Ca}^{2+}$ , preventing  $\text{Ca}^{2+}$  overload [57].

## **4. PREVENTION AND TREATMENT OF MITOCHONDRIAL DYSFUNCTION IN SEVERE SHOCK**

MD is an important factor affecting the occurrence and prognosis of severe shock; therefore, the prevention and treatment of MD has become a new target for the treatment of severe shock. The main therapeutic approaches used to date are shown in Table 1.

### **4.1. Amelioration of Substrate Metabolism**

Adequate substrate provision at complexes I and II and the electron-transferring flavin proteins (ETFs) of the electron transport system (ETS) are linked closely to the function of two other critical upstream metabolic processes that take place in the mitochondria:  $\beta$ -oxidation and the tricarboxylic acid cycle. L-carnitine is essential for the transport of long-chain cytosolic fatty acids into mitochondria for  $\beta$ -oxidation. Succinate is another substrate that may augment oxidative phosphorylation, which fuels complex II of the ETS. ATP-MgCl<sub>2</sub>, a form of exogenous ATP, prevents deleterious hemodynamic effects and protects ATP from deamination. Moreover, the use of mobile electron carriers, such as cytochrome c and ubiquinol (coenzyme Q), has been investigated as another mechanism to restore ETS function. Caffeine (1,3,7-trimethylxanthine) can enhance mitochondrial cyclic adenosine monophosphate (cAMP) as a potential mitochondrial therapy. Alpha-lipoic acid is an organosulfur compound, one enantiomer of which is an essential cofactor for many enzyme complexes, including mitochondrial pyruvate dehydrogenase, an enzyme complex essential for normal aerobic metabolism [4]. Other substances, such as pyruvate and



glucose, enhance the function of mitochondrial oxidative phosphorylation, and increase the production of ATP. However, the effect of these substances is not specifically targeted at mitochondria; they have indirect effects against damaging factors (e.g., ROS release, calcium overload and mPTP opening) on mitochondria. Thus, they do not strictly belong to the group of mitochondria protective agents.

**Table 1. Mitochondria are a potential target in shock treatment**

Mitochondrial target	Drugs
Energy substrate metabolism	L-carnitine succinate ATP-MgCl <sub>2</sub> coenzyme Q $\alpha$ -lipoic acid
ROS production	N-acetylcysteine tempol MitoQ phenyl-tert-butyl nitron ethyl pyruvate
mPTP opening	cyclosporin A NIM811 bongkreikic acid
Iron channel	ruthenium red diazoxide
SIRT1/3	SRT1720 resVida Longevinex® SRT501
Multi-targets	melatonin resveratrol polydatin

ROS, reactive oxygen species; mPTP, mitochondrial permeability transition pore; SIRT1, sirtuin 1; SIRT3, sirtuin 3.

## 4.2. Reduction of Oxidative Stress

To reduce oxidative stress, enhancing ROS scavenging and increasing the anti-oxidative ability are two important factors. As ROS scavengers, glutathione peroxidase (GSH-Px), catalase (CAT), and superoxide dismutase (SOD) are important catalytic enzymes. Glutathione is the most abundant intracellular antioxidant and forms the basis of the mitochondrial defense system. N-acetylcysteine (NAC) is a precursor in the formation of glutathione and is the

N-acetyl derivative of L-cysteine [58]. SOD2 is regarded as a simple scavenging enzyme whose activity is thought to be only dependent stoichiometrically on levels of superoxide in the mitochondria [22, 59, 60]. Notably, a number of electron acceptors and compounds that mimic superoxide dismutase have been developed as mitochondrial therapies in models of both hemorrhagic and septic shock [61, 62]. Tempol, a stable piperidine nitroxide, is one such low-molecular-weight ROS scavenger that can permeate plasma and mitochondrial membranes to reach sites of ROS production [61]. In rodent models of sepsis and multiple organ failure, Tempol lowered peroxynitrite formation and attenuated MD [62]. The administration of Tempol in established sepsis also reduced organ dysfunction and improved survival [62, 63].

To enhance the anti-oxidative effect, one approach to develop an exogenous mitochondrial antioxidant was the use of the cationic compound triphenylphosphonium (TPP<sup>+</sup>). The protective effects of MitoQ, a mitochondrial-targeted composite of ubiquinol, ubiquinone, and a TPP<sup>+</sup> analog, have been proved in isolated mitochondria, cells and animal models of oxidative stress [64]. Another cationic antioxidant, phenyl-tert-butyl nitron (PBN), has protective effects against both endotoxic shock and ischemia–reperfusion injury in animal models. Another promising endogenous compound with ROS scavenging properties is ethyl pyruvate, which is an ester derivative of pyruvate. Ethyl pyruvate has been trialed in a calcium- and potassium-containing balanced salt solution, similar to Ringer's lactate, a commonly employed resuscitation fluid. It is reported that the resuscitation fluid with ethyl pyruvate could improve the animal survival time following hemorrhagic shock [65, 66]; however, a later study by Mulier et al. showed that ethyl pyruvate does not improve early hemodynamics or tissue energetics [67].

### 4.3. Inhibition of mPTP Opening

It is generally accepted that CsA is a mitochondrial protector that targets cyclophilin D (CyPD), a subunit of mPTP, preventing the development of the mPT through membrane stabilization. In addition, CsA reduces the mPTP's sensitivity to Ca<sup>2+</sup> and the combination of CyPD and PiC, thus inhibiting the opening of the mPTP [68]. However, because of the severe immunosuppressive side effects of CsA, new cyclosporin compounds should be developed to achieve mitochondrial protection. Other nonimmuno-suppressive cyclosporin analogs, such as NIM811 [69], and bongkreikic acid (also an inhibitor of the

adenonucleotide transporter, ANT), and antiapoptotic B cell leukemia (Bcl) proteins, have shown similar cytoprotective effects [69].

#### 4.4. Regulation of Ion Channels

Mitochondrial calcium overload can cause mPTP opening and subsequent MD [26]. Ruthenium red, a calcium channel inhibitor, lowers overloaded mitochondrial calcium, thus inhibiting mPTP opening and protecting mitochondria function [31]. Moreover, diazoxide, a mitochondrial ATP sensitive  $K^+$  channel opener, can inhibit calcium overload via the induction of  $K^+$  uptake, which subsequently inhibits mPTP opening [32].

#### 4.5. Activation of SIRT1/3

A growing body of evidence shows that the deacetylase, sirtuin1 (SIRT1), catalyzes the deacetylation of acetyl-lysine residues of proteins such as FOXO3a and SOD2, which protect mitochondria indirectly against shock attack in multiple ways [70]. Therefore, sirtuin-family members and their activators might be promising therapeutic targets for ischemia-reperfusion injury. Interestingly, previous studies and ours confirmed that SIRT1 activation increased the protein expression [21, 71] and activity [72-74] of peroxisome proliferator-activated receptor- $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ), a powerful controller of cell metabolism, and the protein expression of SOD2 [21, 22, 75]. We also showed that SIRT1 protein level and deacetylase activities were decreased in diverse organs (such as arterial smooth muscle, liver and kidney) during severe hemorrhagic shock, which involved the genesis of MD with persistent hypotension and organ damage in severe shock [22, 34, 44, 56, 76]. Notably, p53 is another typical downstream effector of SIRT1. The p53 gene was the first non-histone SIRT1 deacetylation target to be discovered [77, 78]. Numerous studies have demonstrated the deacetylation role of SIRT1 on p53 and the subsequent downregulation of p53 activity [77, 79]. Recently, deacetylation of p53 by SIRT1 activator was observed to reduce cisplatin-induced injury to proximal tubular epithelial cells in mice [80], and doxorubicin induced myocardial apoptosis [81]. Our recent study suggested that decreased SIRT1 activity may result in weaker deacetylation of p53, This causes increased translocation of p53 from the cytoplasm to the mitochondria, accompanied by release of mitochondrial pro-apoptotic proteins, which leads to mitochondrial

damage and apoptosis. Furthermore, other targets of sirtuin should receive attention. SIRT1 can deacetylate pro-inflammation related proteins NF- $\kappa$ B [82] and HMGB-1 [83], which reduces the inflammatory response, which may further prevent MD indirectly. Notably, resVida, Longevinex® and SRT501 have emerged as reformulated versions of RSV, with improved bioavailability, which as SIRT1 activators [84]. In addition to the widely reported RSV and its analogs, SRT1720 is another molecules that is structurally unrelated to RSV. SRT1720 has also been developed to stimulate sirtuin activities more potently than RSV.

In addition to SIRT1, another family member, SIRT3, has emerged as a mitochondrial fidelity protein that directs energy generation, regulates ROS scavenging proteins and protects mitochondria [85, 86]. Loss of function or genetic mutation of these fidelity proteins create a cellular environment that is permissive for the development of MD [87]. Recently, we found that a reduction in both the level and activity of SIRT3 is involved in vascular hyporeactivity following HS [88]. This is accompanied by decreased SOD2 protein content and activity. Moreover, both the protein content and activity were decreased in the small intestine of shocked rats. Collectively, these previous studies indicated that the SIRT1/3-SOD2 axis might exist in the pathogenesis of shock induced MD. Unfortunately, no drug that targets SIRT3 activation directly has been developed, which requires further research [84].

#### **4.6. Administration of Multi-Target Drugs**

There are some drugs that have multiple therapeutic targets; however, they are classified infrequently into the above categories, being listed as “multi-target therapeutic drug administration”. The first drug is melatonin, which is an endogenous antioxidant that has properties that preserve mitochondrial bioenergetics [89]. Melatonin lowers mitochondrial ROS production and stimulates glutathione synthesis and transport [90]. It has direct antagonistic effects on the receptor channel pore N-methyl-D-aspartate, which controls calcium channel opening; thus preventing the calcium influx associated with MD [91]. Exogenous administration of melatonin after a septic insult prevented MD and restored phosphorylation capacity, presumably via its ability to scavenge nitric oxide species, ameliorate lipid peroxidation and associated oxidative damage, and restore glutathione levels [4]. Recently, melatonin was reported to activate SIRT1 activity [92].

RSV, a natural polyphenolic compound, not only inhibits the generation of oxygen free radical, but also chelates ferrous iron, protecting lysosomes against oxidative stress. Based on its stabilization of lysosomes, RSV depresses the release of lysosomal enzymes and apoptosis inducing factors, and, subsequently, phosphorylated GSK-3, leading to inhibition of mPTP opening [93]. Moreover, RSV has a weak anti-inflammatory effect via inactivation of inflammatory cytokines such as NF-kappa B. Recently a hotspot of RSV research has focused on its effect as a novel activator of SIRT1. Based on its activation of SIRT1, RSV deacetylates certain downstream targets mentioned above, leading to anti-oxidative, anti-inflammatory and apoptosis inhibition effects. Thus, we believe that RSV is a promising candidate in MD treatment.

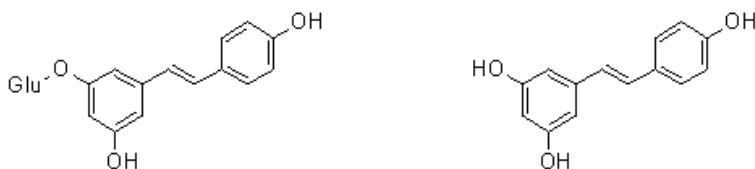


Figure 4. Molecular structure of PD (polydatin) (left) and resveratrol (right).

PD, which is also known RSV glucoside, is a monocrystalline drug that can be isolated from a traditional Chinese herb (*Polygonum cuspidatum*). The molecular composition of PD is 3,4',5-trihydroxystibene-3-monoglucoside (Figure 4). Numerous studies have shown that PD can inhibit the generation of oxygen free radicals [94]. Our previous studies proved that PD administration attenuates MD in arteriolar smooth muscle cells [76], neurons [44], hepatocytes [22] and renal tubular epithelial cells (RTECs) [34] following HS, and ameliorates mitochondria damage of RTECs against sepsis [95]. In some way, PD inhibits the opening of the mPTP [44, 76]; however, the exact mechanism is not fully understood. Notably, we found that PD could partially restore the expression of SOD2 in the small intestine [21] and hepatocytes [22], and, especially, the activity of SOD2 following severe hemorrhagic shock [21]. We also explored that hypothesis that upregulation of SOD2 by PD might rely on its SIRT1 activation. Further study by our research group demonstrated that PD could also restore SIRT3 activity considerably in hemorrhagic shock animals [20]. Interestingly, the administration of PD not only affects SOD2, but also affects p53. We found that PD inhibited the cytoplasmic shuttling of p53 via SIRT1-mediated p53 deacetylation, indicating that PD administration could affect the upstream signaling of MD [34]. Figure 5 summarizes the potential

mechanism of PD/RSV's mitochondrial protection. However, the exact mechanism of PD's function will be explored in a future study.

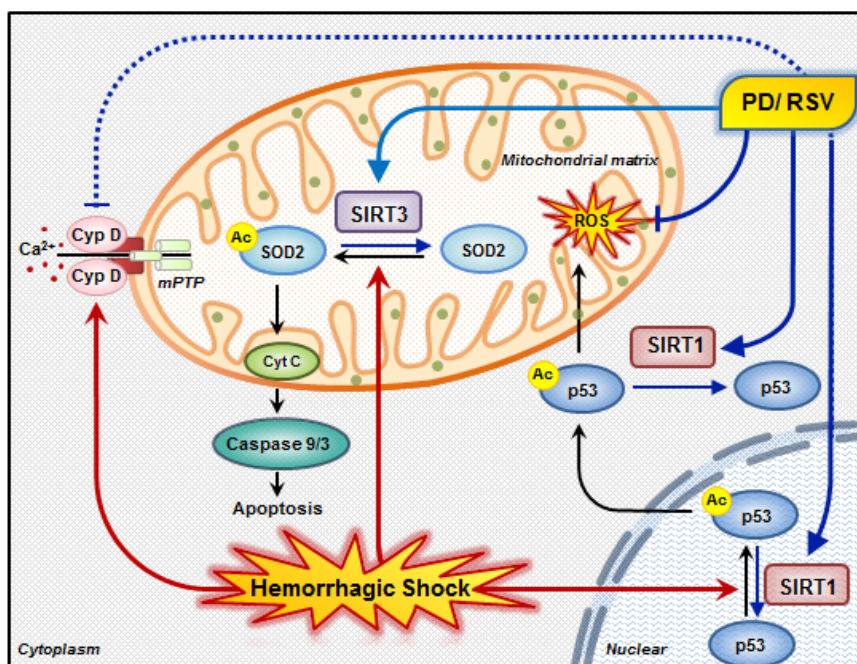


Figure 5. The partial mechanism of mitochondrial dysfunction in hemorrhagic shock (HS) and potential therapeutic targets of PD (polydatin) and resveratrol (RSV). Elevated reactive oxygen species (ROS), reduced anti-oxidative enzyme SOD2, increased pro-apoptotic protein such as p53, and inactivation of SIRT1/3, contribute to the pathogenesis of HS. PD/RSV exert their anti-shock effects through multiple therapeutic targets. On the one hand, PD/RSV directly inhibit the generation of reactive oxygen species (ROS); on the other hand, PD/RSV activate SIRT1/3, deacetylate SOD2 and p53, considerably elevate the anti-oxidative ability of mitochondria and inhibit the mitochondrial apoptosis pathway. In addition, inhibition the opening of the mPTP in mitochondria might be a promising target of PD for MD treatment. ROS, reactive oxygen species; SOD2, superoxide dismutase; Cyp D, cyclophilin D; cytC, cytochrome C; SIRT1, sirtuin 1; SIRT3, sirtuin 3.

## CONCLUSION

MD is a general phenomenon in the pathogenesis of hemorrhagic shock-induced multiple organ injury. Mitochondria are damaged during the response

to severe hemorrhagic shock or ischemia/reperfusion injury. In other cases, the generation of oxidants by mitochondria could induce or amplify tissue dysfunction. However, in some cases, the changes in mitochondria may represent a downstream marker of tissue damage, which may be the actual cause of the refractory hypotension. Experimental studies and some clinical trials have observed MD in multiple cells. We believe that mitochondrial protection is, at least in part, a crucial aspect of severe shock treatment. However, determination of the precise mechanism by further experimental study is needed and more powerful evidence is required for clinical research.

## REFERENCES

- [1] Gutierrez G, Reines HD, Wulf-Gutierrez ME: Clinical review: Hemorrhagic shock. *CRITICAL CARE-LONDON-* (2004) 8(373-381.
- [2] Song R, Bian H, Wang X, Huang X, Zhao K-s: Mitochondrial injury underlies hyporeactivity of arterial smooth muscle in severe shock. *American journal of hypertension* (2011) 24(1):45-51.
- [3] Fink MP: Cytopathic hypoxia: Mitochondrial dysfunction as mechanism contributing to organ dysfunction in sepsis. *Critical care clinics* (2001) 17(1):219-237.
- [4] Dare AJ, Phillips AR, Hickey AJ, Mittal A, Loveday B, Thompson N, Windsor JA: A systematic review of experimental treatments for mitochondrial dysfunction in sepsis and multiple organ dysfunction syndrome. *Free Radical Biology and Medicine* (2009) 47(11):1517-1525.
- [5] Pieczenik SR, Neustadt J: Mitochondrial dysfunction and molecular pathways of disease. *Experimental and molecular pathology* (2007) 83(1):84-92.
- [6] Luft R, Ikkos D, Palmieri G, Ernster L, Afzelius B: A case of severe hypermetabolism of nonthyroid origin with a defect in the maintenance of mitochondrial respiratory control: A correlated clinical, biochemical, and morphological study. *Journal of clinical investigation* (1962) 41(9):1776.
- [7] Miura T, Tanno M: The mptp and its regulatory proteins: Final common targets of signalling pathways for protection against necrosis. *Cardiovascular Research* (2012) 94(2):181-189.
- [8] Kokoszka JE, Waymire KG, Levy SE, Sligh JE, Cai J, Jones DP, MacGregor GR, Wallace DC: The adp/atp translocator is not essential for

- the mitochondrial permeability transition pore. *Nature* (2004) 427(6973):461-465.
- [9] Baines CP, Kaiser RA, Sheiko T, Craigen WJ, Molkenin JD: Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. *Nature cell biology* (2007) 9(5):550-555.
- [10] Leung AW, Varanyuwatana P, Halestrap AP: The mitochondrial phosphate carrier interacts with cyclophilin d and may play a key role in the permeability transition. *Journal of Biological Chemistry* (2008) 283(39):26312-26323.
- [11] Adachi H, Strauss W, Ochi H, Wagner H: The effect of hypoxia on the regional distribution of cardiac output in the dog. *Circulation Research* (1976) 39(3):314-319.
- [12] Schlichtig R, Kramer DJ, Pinsky MR: Flow redistribution during progressive hemorrhage is a determinant of critical o<sub>2</sub> delivery. *Journal of Applied Physiology* (1991) 70(1):169-178.
- [13] Zeng Z, Zhang Q, Gao Y, Li T, Dai X, Huang Q, Chen Z: Drag-reducing polyethylene oxide improves microcirculation after hemorrhagic shock. *Journal of Surgical Research* (2016) 202(1):118-125.
- [14] Weis SN, Pettenuzzo LF, Krolow R, Valentim LM, Mota CS, Dalmaz C, Wyse AT, Netto CA: Neonatal hypoxia-ischemia induces sex-related changes in rat brain mitochondria. *Mitochondrion* (2012) 12(2):271-279.
- [15] Lin C-D, Kao M-C, Tsai M-H, Lai C-H, Wei I-H, Tsai M-H, Tang C-H, Lin C-W, Hsu C-J, Lin C-Y: Transient ischemia/hypoxia enhances gentamicin ototoxicity via caspase-dependent cell death pathway. *Laboratory Investigation* (2011) 91(7):1092-1106.
- [16] Träger K, DeBacker D, Radermacher P: Metabolic alterations in sepsis and vasoactive drug-related metabolic effects. *Current opinion in critical care* (2003) 9(4):271-278.
- [17] Svistunenko DA, Davies N, Brealey D, Singer M, Cooper CE: Mitochondrial dysfunction in patients with severe sepsis: An epr interrogation of individual respiratory chain components. *Biochimica et Biophysica Acta (BBA)-Bioenergetics* (2006) 1757(4):262-272.
- [18] Doise JM, Aho LS, Quenot JP, Guillaud JC, Zeller M, Vergely C, Aube H, Blettery B, Rochette L: Plasma antioxidant status in septic critically ill patients: A decrease over time. *Fundamental and clinical pharmacology* (2008) 22(2):203-209.
- [19] D'Autréaux B, Toledano MB: Ros as signalling molecules: Mechanisms that generate specificity in ros homeostasis. *Nature reviews Molecular cell biology* (2007) 8(10):813-824.



- 
- [20] Zeng Z, Yang Y, Dai X, Xu S, Li T, Zhang Q, Zhao K-s, Chen Z: Polydatin ameliorates injury to the small intestine induced by hemorrhagic shock via sirt3 activation-mediated mitochondrial protection. *Expert Opinion on Therapeutic Targets* (2016) just-accepted).
- [21] Zeng Z, Chen Z, Xu S, Song R, Yang H, Zhao K-s: Polydatin alleviates small intestine injury during hemorrhagic shock as a sirt1 activator. *Oxidative Medicine and Cellular Longevity* (2015) 2015(1-12).
- [22] 22. Wang X, Song R, Bian HN, Ulf T. Brunk, Zhao M, Zhao KS: Polydatin, a natural polyphenol, protects arterial smooth muscle cells against mitochondrial dysfunction and lysosomal destabilization following hemorrhagic shock. *Am J Physiol Regul Integr Comp Physiol* (2012) 302(7):R805-R814.
- [23] Berridge MJ, Taylor C: Inositol trisphosphate and calcium signaling. 53:Abs 927-933.
- [24] Brandman O, Meyer T: Feedback loops shape cellular signals in space and time. *Science* (2008) 322(5900):390-395.
- [25] O'Rourke B: Excitation-contraction coupling and mitochondrial energetics. *Basic research in cardiology* (2007) 102(5):369-392.
- [26] Lemasters JJ, Theruvath TP, Zhong Z, Nieminen A-L: Mitochondrial calcium and the permeability transition in cell death. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* (2009) 1787(11):1395-1401.
- [27] Trollinger DR, Cascio WE, Lemasters JJ: Mitochondrial calcium transients in adult rabbit cardiac myocytes: Inhibition by ruthenium red and artifacts caused by lysosomal loading of  $Ca^{2+}$ -indicating fluorophores. *Biophysical Journal* (2000) 79(1):39-50.
- [28] Schanne F, Kane AB, Young EE, Farber JL: Calcium dependence of toxic cell death: A final common pathway. *Science* (1979) 206(4419):700-702.
- [29] Orrenius S, McConkey DJ, Bellomo G, Nicotera P: Role of  $Ca^{2+}$  in toxic cell killing. *Trends in Pharmacological Sciences* (1989) 10(7):281-285.
- [30] Murata M, Akao M, O'Rourke B, Marbán E: Mitochondrial  $ATP$ -sensitive potassium channels attenuate matrix  $Ca^{2+}$  overload during simulated ischemia and reperfusion possible mechanism of cardioprotection. *Circulation research* (2001) 89(10):891-898.
- [31] Yarana C, Sripetchwandee J, Sanit J, Chattipakorn S, Chattipakorn N: Calcium-induced cardiac mitochondrial dysfunction is predominantly mediated by cyclosporine  $A$ -dependent mitochondrial permeability transition pore. *Archives of medical research* (2012) 43(5):333-338.
- [32] Kupsch K, Parvez S, Siemen D, Wolf G: Modulation of the permeability transition pore by inhibition of the mitochondrial  $K_{ATP}$  channel in liver vs.

- Brain mitochondria. *Journal of Membrane Biology* (2007) 215(2-3):69-74.
- [33] Jeger V, Djafarzadeh S, Jakob SM, Takala J: Mitochondrial function in sepsis. *European journal of clinical investigation* (2013) 43(5):532-542.
- [34] Zeng Z, Chen Z, Xu S, Zhang Q, Wang X, Gao Y, Zhao K-s: Polydatin protecting kidneys against hemorrhagic shock-induced mitochondrial dysfunction via sirt1 activation and p53 deacetylation. *Oxidative Medicine and Cellular Longevity* (2016) 2016(
- [35] Song R, Bian H, Huang X, Zhao Ks: Atractyloside induces low contractile reaction of arteriolar smooth muscle through mitochondrial damage. *Journal of Applied Toxicology* (2012) 32(6):402-408.
- [36] Brand MD, Nicholls DG: Assessing mitochondrial dysfunction in cells. *Biochemical Journal* (2011) 435(2):297-312.
- [37] Zhao KS, Song R, Wang X: Mitochondrial dysfunction in severe hemorrhagic shock. *Advances in Medicine and Biology* (2014) 77:119-133.
- [38] Levy RJ: Mitochondrial dysfunction, bioenergetic impairment, and metabolic down-regulation in sepsis. *Shock* (2007) 28(1):24-28.
- [39] Szabó C, Salzman AL: Inhibition of atp-activated potassium channels exerts pressor effects and improves survival in a rat model of severe hemorrhagic shock. *Shock* (1996) 5(6):391-394.
- [40] Crouser ED: Mitochondrial dysfunction in septic shock and multiple organ dysfunction syndrome. *Mitochondrion* (2004) 4(5):729-741.
- [41] Crouser ED, Julian MW, Blaho DV, Pfeiffer DR: Endotoxin-induced mitochondrial damage correlates with impaired respiratory activity. *Critical care medicine* (2002) 30(2):276-284.
- [42] Cour M, Loufouat J, Paillard M, Augeul L, Goudable J, Ovize M, Argaud L: Inhibition of mitochondrial permeability transition to prevent the post-cardiac arrest syndrome: A pre-clinical study. *European heart journal* (2011) 32(2):226-235.
- [43] Fink MP: Administration of exogenous cytochrome c as a novel approach for the treatment of cytopathic hypoxia\*. *Critical care medicine* (2007) 35(9):2224-2225.
- [44] Wang X, Song R, Chen Y, Zhao M, Zhao KS: Polydatin--a new mitochondria protector for acute severe hemorrhagic shock treatment. *Expert opinion on investigational drugs* (2013) 22(2):169-179.
- [45] Halestrap AP, Clarke SJ, Javadov SA: Mitochondrial permeability transition pore opening during myocardial reperfusion—a target for cardioprotection. *Cardiovascular research* (2004) 61(3):372-385.

- [46] Petronilli V, Miotto G, Canton M, Brini M, Colonna R, Bernardi P, Di Lisa F: Transient and long-lasting openings of the mitochondrial permeability transition pore can be monitored directly in intact cells by changes in mitochondrial calcein fluorescence. *Biophysical journal* (1999) 76(2):725-734.
- [47] Jones RA, Smail A, Wilson MR: Detecting mitochondrial permeability transition by confocal imaging of intact cells pinocytically loaded with calcein. *European Journal of Biochemistry* (2002) 269(16):3990-3997.
- [48] Adrie C, Bachelet M, Vayssier-Taussat M, Russo-Marie F, Bouchaert I, Adib-Conquy M, Cavaillon J-M, Pinsky MR, Dhainaut J-F, Polla BS: Mitochondrial membrane potential and apoptosis peripheral blood monocytes in severe human sepsis. *American journal of respiratory and critical care medicine* (2001) 164(3):389-395.
- [49] Salvioi S, Ardizzoni A, Franceschi C, Cossarizza A: Jc-1, but not dioc 6 (3) or rhodamine 123, is a reliable fluorescent probe to assess  $\delta\psi$  changes in intact cells: Implications for studies on mitochondrial functionality during apoptosis. *FEBS letters* (1997) 411(1):77-82.
- [50] Zhao K-s, Huang X, Liu J, Huang Q, Jin C, Jiang Y, Jin J, Zhao G: New approach to treatment of shock—restitution of vasoreactivity. *Shock* (2002) 18(2):189-192.
- [51] Kon K, Kim J-S, Uchiyama A, Jaeschke H, Lemasters JJ: Lysosomal iron mobilization and induction of the mitochondrial permeability transition in acetaminophen-induced toxicity to mouse hepatocytes. *Toxicological Sciences* (2010) kfq175.
- [52] Zhao M, Antunes F, Eaton JW, Brunk UT: Lysosomal enzymes promote mitochondrial oxidant production, cytochrome c release and apoptosis. *European Journal of Biochemistry* (2003) 270(18):3778-3786.
- [53] Javadov S, Karmazyn M: Mitochondrial permeability transition pore opening as an endpoint to initiate cell death and as a putative target for cardioprotection. *Cellular Physiology and Biochemistry* (2007) 20(1-4):1-22.
- [54] Szewczyk A, Wojtczak L: Mitochondria as a pharmacological target. *Pharmacological reviews* (2002) 54(1):101-127.
- [55] Morin D, Papadopoulos V, Tillement J-P: Prevention of cell damage in ischaemia: Novel molecular targets in mitochondria. *Expert Opinion on Therapeutic Targets* (2002) 6(3):315-334.
- [56] Zeng Z, Chen Z, Li T, Zhang J, Gao Y, Xu S, Cai S, Zhao K-s: Polydatin: A new therapeutic agent against multiorgan dysfunction. *Journal of Surgical Research* (2015) 198(1):192-199.

- 
- [57] Garlid KD, Paucek P, Yarov-Yarovoy V, Murray HN, Darbenzio RB, D'Alonzo AJ, Lodge NJ, Smith MA, Grover GJ: Cardioprotective effect of diazoxide and its interaction with mitochondrial atp-sensitive k<sup>+</sup> channels possible mechanism of cardioprotection. *Circulation research* (1997) 81(6):1072-1082.
- [58] Portella A, Montero E, de Figueiredo LP, Bueno A, Thurow A, Rodrigues F: Effects of n-acetylcysteine in hepatic ischemia-reperfusion injury during hemorrhagic shock. 36:Abs 846-848.
- [59] Qiu X, Brown K, Hirschey MD, Verdin E, Chen D: Calorie restriction reduces oxidative stress by sirt3-mediated sod2 activation. *Cell Metab* (2010) 12(6):662-667.
- [60] Pi H, Xu S, Reiter RJ, Guo P, Zhang L, Li Y, Li M, Cao Z, Tian L, Xie J, Zhang R *et al*: Sirt3-sod2-mros-dependent autophagy in cadmium-induced hepatotoxicity and salvage by melatonin. *Autophagy* (2015) 11(7):1037-1051.
- [61] Fink MP, Macias CA, Xiao J, Tyurina YY, Delude RL, Greenberger JS, Kagan VE, Wipf P: Hemigramicidin-tempo conjugates: Novel mitochondria-targeted antioxidants. *Crit Care Med* (2007) 35(9 Suppl):S461-467.
- [62] Macias CA, Chiao JW, Xiao J, Arora DS, Tyurina YY, Delude RL, Wipf P, Kagan VE, Fink MP: Treatment with a novel hemigramicidin-tempo conjugate prolongs survival in a rat model of lethal hemorrhagic shock. *Ann Surg* (2007) 245(2):305-314.
- [63] Liaw WJ, Chen TH, Lai ZZ, Chen SJ, Chen A, Tzao C, Wu JY, Wu CC: Effects of a membrane-permeable radical scavenger, tempol, on intraperitoneal sepsis-induced organ injury in rats. *Shock* (2005) 23(1):88-96.
- [64] Adlam VJ, Harrison JC, Porteous CM, James AM, Smith RA, Murphy MP, Sammut IA: Targeting an antioxidant to mitochondria decreases cardiac ischemia-reperfusion injury. *The FASEB Journal* (2005) 19(9):1088-1095.
- [65] Tawadrous ZS, Delude RL, Fink MP: Resuscitation from hemorrhagic shock with ringer's ethyl pyruvate solution improves survival and ameliorates intestinal mucosal hyperpermeability in rats. *Shock* (2002) 17(6):473-477.
- [66] Fink MP: Ringer's ethyl pyruvate solution: A novel resuscitation fluid for the treatment of hemorrhagic shock and sepsis. *Journal of Trauma-Injury Infection and Critical Care* (2003) 54(5):S141.

- [67] Mulier KE, Beilman GJ, Conroy MJ, Taylor JH, Skarda DE, Hammer BE: Ringer's ethyl pyruvate in hemorrhagic shock and resuscitation does not improve early hemodynamics or tissue energetics. *Shock* (2005) 23(3):248-252.
- [68] Hausenloy DJ, Boston-Griffiths E, Yellon D: Cyclosporin a and cardioprotection: From investigative tool to therapeutic agent. *British journal of pharmacology* (2012) 165(5):1235-1245.
- [69] Larche J, Lancel S, Hassoun SM, Favory R, Decoster B, Marchetti P, Chopin C, Neviere R: Inhibition of mitochondrial permeability transition prevents sepsis-induced myocardial dysfunction and mortality. *Journal of the American College of Cardiology* (2006) 48(2):377-385.
- [70] Han M-K, Song E-K, Guo Y, Ou X, Mantel C, Broxmeyer HE: Sirt1 regulates apoptosis and nanog expression in mouse embryonic stem cells by controlling p53 subcellular localization. *Cell stem cell* (2008) 2(3):241-251.
- [71] Aquilano K, Baldelli S, Pagliei B, Cannata SM, Rotilio G, Ciriolo MR: P53 orchestrates the pgc-1 $\alpha$ -mediated antioxidant response upon mild redox and metabolic imbalance. *Antioxidants and redox signaling* (2013) 18(4):386-399.
- [72] Nemoto S, Fergusson MM, Finkel T: Sirt1 functionally interacts with the metabolic regulator and transcriptional coactivator pgc-1 {alpha}. *J Biol Chem* (2005) 280(16):16456-16460.
- [73] Iwabu M, Yamauchi T, Okada-Iwabu M, Sato K, Nakagawa T, Funata M, Yamaguchi M, Namiki S, Nakayama R, Tabata M, Ogata H *et al*: Adiponectin and adipor1 regulate pgc-1 $\alpha$  and mitochondria by ca(2+) and ampk/sirt1. *Nature* (2010) 464(7293):1313-1319.
- [74] Rodgers JT, Lerin C, Haas W, Gygi SP, Spiegelman BM, Puigserver P: Nutrient control of glucose homeostasis through a complex of pgc-1 $\alpha$  and sirt1. *Nature* (2005) 434(7029):113-118.
- [75] Kang C, Li Ji L: Role of pgc-1 $\alpha$  signaling in skeletal muscle health and disease. *Annals of the New York Academy of Sciences* (2012) 1271(1):110-117.
- [76] Wang X, Song R, Bian HN, Brunk UT, Zhao M, Zhao K-s: Polydatin, a natural polyphenol, protects arterial smooth muscle cells against mitochondrial dysfunction and lysosomal destabilization following hemorrhagic shock. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* (2012) 302(7):R805-R814.

- 
- [77] Vaziri H, Dessain SK, Eaton EN, Imai S-I, Frye RA, Pandita TK, Guarente L, Weinberg RA: Hsir2sirt1 functions as an nad-dependent p53 deacetylase. *Cell* (2001) 107(2):149-159.
- [78] Luo J, Nikolaev AY, Imai S-i, Chen D, Su F, Shiloh A, Guarente L, Gu W: Negative control of p53 by sir2 $\alpha$  promotes cell survival under stress. *Cell* (2001) 107(2):137-148.
- [79] Langley E, Pearson M, Faretta M, Bauer UM, Frye RA, Minucci S, Pelicci PG, Kouzarides T: Human sir2 deacetylates p53 and antagonizes pml/p53-induced cellular senescence. *The EMBO journal* (2002) 21(10):2383-2396.
- [80] Kim DH, Jung YJ, Lee JE, Lee AS, Kang KP, Lee S, Park SK, Han MK, Lee SY, Ramkumar KM: Sirt1 activation by resveratrol ameliorates cisplatin-induced renal injury through deacetylation of p53. *American Journal of Physiology-Renal Physiology* (2011) 301(2):F427-F435.
- [81] Zhang C, Feng Y, Qu S, Wei X, Zhu H, Luo Q, Liu M, Chen G, Xiao X: Resveratrol attenuates doxorubicin-induced cardiomyocyte apoptosis in mice through sirt1-mediated deacetylation of p53. *Cardiovascular research* (2011) 90(3):538-545.
- [82] Shen Z, Ajmo JM, Rogers CQ, Liang X, Le L, Murr MM, Peng Y, You M: Role of sirt1 in regulation of lps-or two ethanol metabolites-induced tnf- $\alpha$  production in cultured macrophage cell lines. *American Journal of Physiology-Gastrointestinal and Liver Physiology* (2009) 296(5):G1047-G1053.
- [83] Rickenbacher A, Jang JH, Limani P, Ungethüm U, Lehmann K, Oberkofler CE, Weber A, Graf R, Humar B, Clavien P-A: Fasting protects liver from ischemic injury through sirt1-mediated downregulation of circulating hmgb1 in mice. *Journal of hepatology* (2014) 61(2):301-308.
- [84] Villalba JM, Alcáin FJ: Sirtuin activators and inhibitors. *BioFactors* (2012) 38(5):349-359.
- [85] Pillai VB, Sundareshan NR, Jeevanandam V, Gupta MP: Mitochondrial sirt3 and heart disease. *Cardiovasc Res* (2010) 88(2):250-256.
- [86] Chen Y, Zhang J, Lin Y, Lei Q, Guan KL, Zhao S, Xiong Y: Tumour suppressor sirt3 deacetylates and activates manganese superoxide dismutase to scavenge ros. *EMBO Rep* (2011) 12(6):534-541.
- [87] Tao R, Vassilopoulos A, Parisiadou L, Yan Y, Gius D: Regulation of mnsod enzymatic activity by sirt3 connects the mitochondrial acetylome signaling networks to aging and carcinogenesis. *Antioxidants and Redox Signaling* (2014) 20(10):1646-1654.

- [88] Li P, Meng X, Bian H, Burns N, Zhao KS, Song R: Activation of sirtuin 1/3 improves vascular hyporeactivity in severe hemorrhagic shock by alleviation of mitochondrial damage. *Oncotarget* (2015) 6(35):36998-37011.
- [89] Leon J, Acuña-Castroviejo D, Escames G, Tan DX, Reiter RJ: Melatonin mitigates mitochondrial malfunction. *Journal of pineal research* (2005) 38(1):1-9.
- [90] Martín M, Macías M, Escames G, León J, Acuña-Castroviejo D: Melatonin but not vitamins c and e maintains glutathione homeostasis in t-butyl hydroperoxide-induced mitochondrial oxidative stress. *The FASEB Journal* (2000) 14(12):1677-1679.
- [91] Escames G, Macias M, Leon J, Garcia J, Khaldy H, Martin M, Vives F, Acuña-Castroviejo D: Calcium-dependent effects of melatonin inhibition of glutamatergic response in rat striatum. *Journal of neuroendocrinology* (2001) 13(5):459-466.
- [92] Yang Y, Jiang S, Dong Y, Fan C, Zhao L, Yang X, Li J, Di S, Yue L, Liang G: Melatonin prevents cell death and mitochondrial dysfunction via a sirt1-dependent mechanism during ischemic-stroke in mice. *Journal of pineal research* (2015) 58(1):61-70.
- [93] Xi J, Wang H, Mueller RA, Norfleet EA, Xu Z: Mechanism for resveratrol-induced cardioprotection against reperfusion injury involves glycogen synthase kinase 3 $\beta$  and mitochondrial permeability transition pore. *European journal of pharmacology* (2009) 604(1):111-116.
- [94] Ji H, Zhang X, Du Y, Liu H, Li S, Li L: Polydatin modulates inflammation by decreasing nf-kb activation and oxidative stress by increasing gli1, ptch1, sod1 expression and ameliorates blood-brain barrier permeability for its neuroprotective effect in pmcao rat brain. *Brain research bulletin* (2012) 87(1):50-59.
- [95] Gao Y, Zeng Z, Li T, Xu S, Wang X, Chen Z, Lin C: Polydatin inhibits mitochondrial dysfunction in the renal tubular epithelial cells of a rat model of sepsis-induced acute kidney injury. *Anesthesia and analgesia* (2015) 121(5):1251-1260.

## BIOGRAPHICAL SKETCHES

*Zhenhua Zeng*

**Name:** Zhenhua Zeng

**Affiliation:**

Department of Critical Care Medicine, Nanfang Hospital, Southern Medical University, Guangzhou, China

**Education:**

Ph.D. of Southern Medical University, China

**Business Address:**

Department of Critical Care Medicine, Nanfang Hospital, Southern Medical University, Guangzhou 510515, China

**Research and Professional Experience:**

Focus the mechanic research of shock and potential therapeutic targets of shock treatment since 2010 in Guangdong Key Lab of Shock and Microcirculation Research, Department of Pathophysiology, Southern Medical University.

**Professional Appointments:**

None

**Honors:**

None

**Publications Last 3 Years:**

1. Zeng Z, Chen Z, Xu S, Zhang Q, Wang X, Gao Y, Zhao K: Polydatin Protecting Kidneys against Hemorrhagic Shock-Induced Mitochondrial Dysfunction via SIRT1 Activation and p53 Deacetylation. *OXID MED CELL LONGEV* 2016, 2016:1-15.
2. Zeng Z, Yang Y, Dai X, Xu S, Li T, Zhang Q, Zhao K, Chen Z: Polydatin ameliorates injury to the small intestine induced by hemorrhagic shock via SIRT3 activation-mediated mitochondrial protection. *EXPERT OPIN THER TAR* 2016(just-accepted).
3. Qin Z, Zeng Z: Anaphylaxis to chlorhexidine in a chlorhexidine-coated central venous catheter during general anaesthesia. *Anaesth Intensive Care* 2016, 44(2):297-298.
4. Zeng Z, Zhang Q, Gao Y, Li T, Dai X, Huang Q, Chen Z: Drag-reducing polyethylene oxide improves microcirculation after hemorrhagic shock. *J SURG RES* 2016, 202(1):118-125.



5. Dai X, Zeng Z, Fu C, Zhang SA, Cai Y, Chen Z: Diagnostic value of neutrophil gelatinase-associated lipocalin, cystatin C, and soluble triggering receptor expressed on myeloid cells-1 in critically ill patients with sepsis-associated acute kidney injury. *CRIT CARE* 2015, 19(1).
6. Zeng Z, Chen Z, Li T, Zhang J, Gao Y, Xu S, Cai S, Zhao K: Polydatin: a new therapeutic agent against multiorgan dysfunction. *J SURG RES* 2015, 198(1):192-199.
7. Zeng Z, Chen Z, Xu S, Song R, Yang H, Zhao K: Polydatin Alleviates Small Intestine Injury during Hemorrhagic Shock as a SIRT1 Activator. *OXID MED CELL LONGEV* 2015, 2015:1-12.
8. Gao Y, Zeng Z, Li T, Xu S, Wang X, Chen Z, Lin C: Polydatin Inhibits Mitochondrial Dysfunction in the Renal Tubular Epithelial Cells of a Rat Model of Sepsis-Induced Acute Kidney Injury. *Anesthesia and Analgesia* 2015, 121(5):1251-1260.
9. Lin B, Liu Y, Li T, Zeng K, Cai S, Zeng Z, Lin C, Chen Z, Gao Y: Ulinastatin mediates protection against vascular hyperpermeability following hemorrhagic shock. *Int J Clin Exp Pathol* 2015, 8(7):7685-7693.
10. Li T, Liu Y, Li G, Wang X, Zeng Z, Cai S, Li F, Chen Z: Polydatin attenuates ipopolysaccharide-induced acute lung injury in rats. *Int J Clin Exp Pathol* 2014, 7(12):8401-8410.
11. Li T, Cai S, Zeng Z, Zhang J, Gao Y, Wang X, Chen Z: Protective effect of polydatin against burn-induced lung injury in rats. *Respir Care* 2014, 59(9):1412-1421.
12. Li G, Li T, Li Y, Cai S, Zhang Z, Zeng Z, Wang X, Gao Y, Li Y, Chen Z: Ulinastatin inhibits oxidant-induced endothelial hyperpermeability and apoptotic signaling. *INT J CLIN EXP PATHO* 2014, 7(11):7342-7350.

### *Siqi Xu*

**Name:** Siqi Xu

**Affiliation:**

Department of Pathophysiology, Southern Medical University, Guangzhou, China

**Education:**

Ph.D. of Southern Medical University, China

**Business Address:**

Guangdong Key Lab of Shock and Microcirculation Research, Department of Pathophysiology, Southern Medical University, Guangzhou 510515, China

**Research and Professional Experience:**

Focus the mechanic research of shock and potential therapeutic targets of shock treatment since 2012 in Guangdong Key Lab of Shock and Microcirculation Research, Department of Pathophysiology, Southern Medical University.

**Professional Appointments:**

None

**Honors:**

None

**Publications Last 3 Years:**

1. Zeng Z, Chen Z, Xu S, Song R, Yang H, Zhao K: Polydatin Alleviates Small Intestine Injury during Hemorrhagic Shock as a SIRT1 Activator. *OXID MED CELL LONGEV* 2015, 2015:1-12.
2. Zeng Z, Yang Y, Dai X, Xu S, Li T, Zhang Q, Zhao K, Chen Z: Polydatin ameliorates injury to the small intestine induced by hemorrhagic shock via SIRT3 activation-mediated mitochondrial protection. *EXPERT OPIN THER TAR* 2016(just-accepted).
3. Gao Y, Zeng Z, Li T, Xu S, Wang X, Chen Z, Lin C: Polydatin Inhibits Mitochondrial Dysfunction in the Renal Tubular Epithelial Cells of a Rat Model of Sepsis-Induced Acute Kidney Injury. *Anesthesia and Analgesia* 2015, 121(5):1251-1260.
4. Zeng Z, Chen Z, Xu S, Zhang Q, Wang X, Gao Y, Zhao K: Polydatin Protecting Kidneys against Hemorrhagic Shock-Induced Mitochondrial Dysfunction via SIRT1 Activation and p53 Deacetylation. *OXID MED CELL LONGEV* 2016, 2016:1-15.
5. Zeng Z, Chen Z, Li T, Zhang J, Gao Y, Xu S, Cai S, Zhao K: Polydatin: a new therapeutic agent against multiorgan dysfunction. *J SURG RES* 2015, 198(1):192-199.

***Ke-seng Zhao***

**Name:** Ke-seng Zhao

**Affiliation:**

Department of Pathophysiology, Southern Medical University, Guangzhou, China

**Education:**

M.B. of Harbin Medical University, China

**Business Address:**

Guangdong Key Lab of Shock and Microcirculation Research, Department of Pathophysiology, Southern Medical University, Guangzhou 510515, China

**Research and Professional Experience:**

Focus the mechanic research of shock and potential therapeutic targets of shock treatment since 2012 in Guangdong Key Lab of Shock and Microcirculation Research, Department of Pathophysiology, Southern Medical University.

**Professional Appointments:**

Professor of Department of Pathophysiology, Southern Medical University

**Honors:**

None

**Publications Last 3 Years[1-6]:**

1. Zeng Z, Yang Y, Dai X, Xu S, Li T, Zhang Q, Zhao K, Chen Z: Polydatin ameliorates injury to the small intestine induced by hemorrhagic shock via SIRT3 activation-mediated mitochondrial protection. EXPERT OPIN THER TAR 2016, 20(6):645-652.
2. Li P, Meng X, Bian H, Burns N, Zhao K, Song R: Activation of sirtuin 1/3 improves vascular hyporeactivity in severe hemorrhagic shock by alleviation of mitochondrial damage. ONCOTARGET 2015, 6(35):36998-37011.
3. Zeng Z, Chen Z, Xu S, Zhang Q, Wang X, Gao Y, Zhao K: Polydatin Protecting Kidneys against Hemorrhagic Shock-Induced Mitochondrial

---

Dysfunction via SIRT1 Activation and p53 Deacetylation. *OXID MED CELL LONGEV* 2016, 2016:1737185.

4. Zeng Z, Chen Z, Li T, Zhang J, Gao Y, Xu S, Cai S, Zhao K: Polydatin: a new therapeutic agent against multiorgan dysfunction. *The Journal of surgical research* 2015, 198(1):192-199.
5. Li P, Wang X, Zhao M, Song R, Zhao K: Polydatin protects hepatocytes against mitochondrial injury in acute severe hemorrhagic shock via SIRT1-SOD2 pathway. In: *Expert Opin Ther Targets*. 2015 Jul;19(7):997-1010. doi: 10.1517/14728222.2015.1054806., vol. 19; 2015: 997-1010.
6. Zeng Z, Yang Y, Dai X, Xu S, Li T, Zhang Q, Zhao K, Chen Z: Polydatin ameliorates injury to the small intestine induced by hemorrhagic shock via SIRT3 activation-mediated mitochondrial protection. *EXPERT OPIN THER TAR* 2016(just-accepted).



**Chapter 4**

# **TELOMERASE-TARGETED REPLICATIVE ADENOVIRUSES FOR CANCER TREATMENT AND DIAGNOSIS**

***Hiroshi Tazawa<sup>1,2,\*</sup>, Shunsuke Kagawa<sup>1,3</sup>,  
Kunitoshi Shigeyasu<sup>1</sup> and Toshiyoshi Fujiwara<sup>1</sup>***

<sup>1</sup>Department of Gastroenterological Surgery, Okayama University  
Graduate School of Medicine, Dentistry and Pharmaceutical Sciences,  
Okayama, Japan

<sup>2</sup>Center for Innovative Clinical Medicine

<sup>3</sup>Center for Minimally Invasive Therapy, Okayama University Hospital,  
Okayama, Japan

## **ABSTRACT**

Oncolytic virotherapy has recently emerged as a promising antitumor strategy in which tumor-specific cell death is induced by an oncolytic virus. To induce such tumor-specific cell death, tumor specificity for virus replication or infection is needed. Since tumor cells possess unlimited proliferation ability through activation of telomerase, which elongates chromosomal telomeres and prevents the induction of cell cycle arrest and senescence, telomerase activity is a tumor-specific target molecule for the

---

\* Address correspondence to this author at Center for Innovative Clinical Medicine, Okayama University Hospital, 2-5-1 Shikata-cho, Okayama 700-8558, Japan, Phone: 81-86-235-7491; Fax: 81-86-235-7492; E-mail: htazawa@md.okayama-u.ac.jp.

development of an oncolytic virus. We recently generated two types of telomerase-specific replication-competent oncolytic adenoviruses, OBP-301 (Telomelysin) and OBP-401 (TelomeScan) for cancer treatment and diagnosis, respectively. When OBP-301 infects tumor cells, it induces cell lysis. On the other hand, when OBP-401 infects tumor cells, it induces the expression of green fluorescence protein (GFP). This chapter focuses on the recent advances in evaluating the therapeutic potential of OBP-301 in oncolytic virotherapy and the diagnostic potential of OBP-401 for tumor cell detection systems in preclinical and clinical settings. The potential application of OBP-301-based oncolytic virotherapy is discussed in combination therapy with conventional chemotherapy and radiotherapy. The fluorescence-guided tumor detection system using OBP-401 is discussed in terms of the analysis of circulating tumor cells (CTC). A better understanding of the precise molecular mechanism of the OBP-301-mediated tumor suppression system would provide novel insights for the improvement of OBP-301-based oncolytic virotherapy. Furthermore, an OBP-401-based system for capture of GFP-positive CTC in blood samples would improve the assessment of patients with premetastatic advanced cancers.

**Keywords:** telomerase, hTERT, adenovirus, virotherapy, CTC

## ABBREVIATIONS

GFP	green fluorescence protein;
CTC	circulating tumor cells;
hTERT	human telomerase reverse transcriptase;
CAR	coxsackie and adenovirus receptor;
TEP1	telomerase-associated protein 1;
HSP90	heat shock protein 90 kDa;
hTR	human telomerase RNA component;
EGFR	epidermal growth factor receptor;
MCL1	myeloid cell leukemia 1;
DSBs	DNA double strand-breaks;
MRN	Mre11-Rad50-NBS1;
ATM	ataxiatangiectasia mutated;
Vp	virus particles

## INTRODUCTION

Oncolytic virotherapy has recently emerged as a promising antitumor strategy that eliminates tumor cells without adversely affecting surrounding normal cells. Various types of oncolytic viruses are used in oncolytic virotherapy including adenovirus, herpes simplex virus, measles virus, reovirus, and Newcastle disease virus [1, 2]. Among these viruses, adenovirus is one of the most frequently used virus vectors in oncolytic virotherapy.

Oncolytic adenovirus induces tumor-specific cell death through various mechanisms. There are mainly two approaches for the induction of tumor-specific cell death by oncolytic adenovirus. One approach is to induce tumor-specific virus replication. Tumor-specific replication-competent oncolytic adenoviruses are being developed as novel anticancer therapeutic reagents, in which the promoters of cancer-related genes are used to regulate virus replication in a tumor-dependent manner [3-6]. After infection of target cells, virus replication is mainly regulated by the expression level of viral genes. Induction of the adenoviral early *E1* gene is necessary for replication of the adenovirus because an *E1*-deleted adenovirus has no replication ability. Therefore, to induce replication of the oncolytic adenovirus in tumor cells, the wild-type *E1* promoter is genetically modified by inserting several kinds of tumor-specific promoters, such as the human telomerase reverse transcriptase (hTERT) promoter [7-11], midkine promoter [12, 13], cyclooxygenase-2 promoter [14], and survivin promoter [15]. Since the *hTERT* gene is frequently overexpressed in a variety of malignant tumor cells with telomerase activity, the hTERT promoter is a useful tool for the tumor-specific regulation of viral gene expression [16]. We therefore generated a hTERT promoter-driven replication-competent oncolytic adenovirus OBP-301 (Telomelysin) for novel anticancer treatment [11]. The hTERT promoter was a useful tool not only for induction of tumor-specific replication and the oncolytic effect of OBP-301 [17], but also for enhancement of the antitumor activity of OBP-301 even in a hypoxic tumor microenvironment [18]. Another approach for antitumor virotherapy is to induce tumor-specific virus internalization. Adenovirus can enter into target cells through binding to the coxsackie and adenovirus receptor (CAR) protein on their cell surface. Since a tumor-specific surface antigen that is expressed only on tumor cells, and not on normal cells, is a promising target molecule for tumor-specific virus infection, modification of the adenoviral capsid protein binding to a tumor-specific antigen is a useful strategy for



enhancement of the tumor tropism of an adenovirus. Surface modification of an adenovirus vector by genetic, chemical, or mechanical engineering methods has been shown to enhance the infection ability of an adenovirus [19]. Moreover, the development of a tumor-specific delivery system using various types of carrier cells is also a useful method for tumor-specific internalization of an oncolytic adenovirus [20]. Thus, the establishment of tumor specificity for virus replication and internalization is a promising strategy for induction of tumor-specific cell death in oncolytic virotherapy.

A tumor-specific oncolytic virus is a useful tool not only for induction of cell death, but also for induction of the accumulation of an exogenous marker protein in tumor cells. For example, tumor-specific induction of green fluorescence protein (GFP) can be applied for cancer diagnosis. Using a hTERT promoter-driven oncolytic adenovirus OBP-301 as a starting point, we further developed a telomerase-specific replication-competent oncolytic adenovirus OBP-401 (TelomeScan) that induces GFP expression in both epithelial and mesenchymal types of malignant tumor cells with telomerase activity [21, 22]. Intratumoral injection of OBP-401 can permit visualization of residual GFP-positive tumor cells within normal tissues, such as lymph node [21, 23], liver [24], and the peritoneal cavity [25], in *in vivo* animal experiments. Moreover, the small number of circulating tumor cells (CTC) can be detected by OBP-401 as GFP-positive cells in blood samples from patients with various types of cancers [26, 27]. These accumulating evidences have shown that an OBP-401-mediated CTC detection system is broadly acceptable for the assessment of cancer patients with premetastatic CTC. Thus, the tumor-specific accumulation of a fluorescent protein by using an oncolytic virus-based gene transfer system would be a useful strategy for the detection of residual tumor cells in a variety of organs and blood.

This chapter focuses on the recent advances in evaluating the therapeutic potential of the hTERT promoter-driven oncolytic adenovirus OBP-301 in oncolytic virotherapy and the diagnostic potential of the hTERT promoter-driven GFP-expressing oncolytic adenovirus OBP-401 as a tumor detection system in preclinical and clinical settings. The potential application of OBP-301-based oncolytic virotherapy will be especially discussed in terms of combination therapy with conventional chemotherapy and radiotherapy. The fluorescence-guided tumor detection system using OBP-401 will be discussed in terms of analysis of CTC using blood samples from cancer patients.

## ROLE OF TELOMERASE ACTIVITY IN CANCER

### Telomerase-Mediated Unlimited Cell Proliferation

Normal cells undergo cell cycle arrest and senescence-related cell death after cell division because of shortening of the chromosomal telomere end, which is a region of repeated TTAGGG nucleotides (Figure 1). In contrast, even after cell division, cancer cells exhibit unlimited cell proliferation because of telomere elongation caused by activation of telomerase (Figure 1).

Telomerase is an enzyme that adds the TTAGGG repeated sequence to the end of a chromosome. Telomerase is a ribonucleoprotein complex containing two subunits, a catalytic subunit (hTERT, telomerase-associated protein 1 (TEP1), heat shock protein 90 kDa (Hsp90), and p23) and an RNA subunit (human telomerase RNA component (hTR)) (Figure 1). Since more than 80% of human cancer tissues have high telomerase activity, which is highly associated with the expression level of the *hTERT* gene, telomerase activity is a critical target molecule for cancer treatment and diagnosis.

### Telomerase-Targeted Replicative Adenoviruses

We generated two types of telomerase-specific replication-competent oncolytic adenoviruses, OBP-301 (Telomelysin) and OBP-401 (TelomeScan) for cancer treatment and diagnosis, respectively. OBP-301 drives expression of the adenoviral *E1A* and *E1B* genes under the control of the hTERT promoter for tumor-specific virus replication [11]. When tumor cells are infected with OBP-301, OBP-301 induces oncolytic cell lysis in tumor cells with telomerase activities. OBP-401 is a modified OBP-301 that induces GFP expression in tumor cells during virus replication [21, 22]. When tumor cells are infected with OBP-401, OBP-401 induces GFP expression in tumor cells with telomerase activities. Next, we address the recent advances in evaluating the therapeutic potential of OBP-301 in oncolytic virotherapy and the diagnostic potential of OBP-401 as a tumor cell detection system in preclinical and clinical settings.

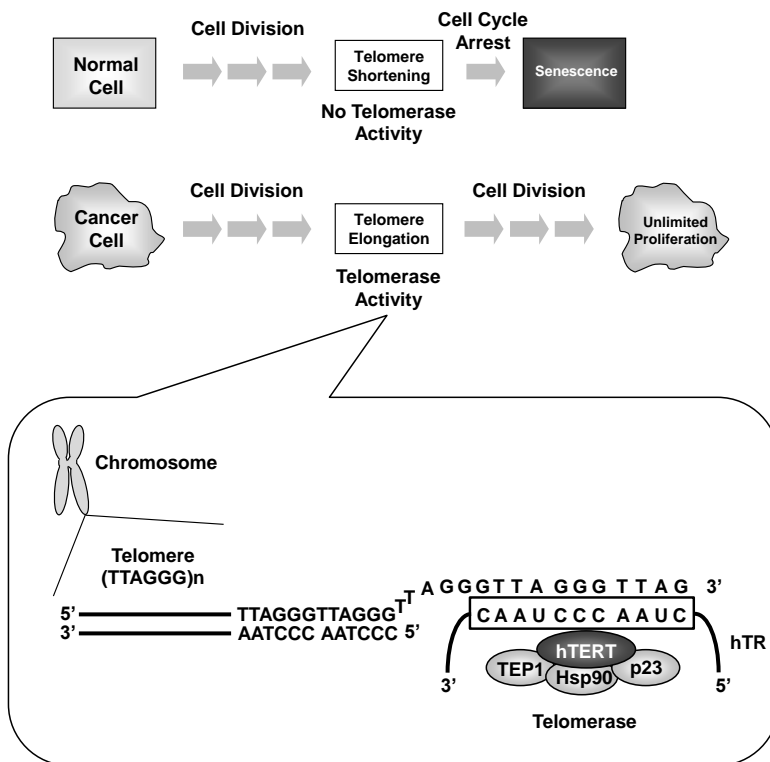


Figure 1. Telomerase-mediated unlimited cell proliferation in tumor cells. Normal cells without telomerase activity undergo cell cycle arrest and senescence after cell division due to telomere shortening. In contrast, cancer cells with telomerase activity undergo unlimited cell proliferation after cell division due to telomerase-mediated telomere elongation. Telomerase consists of a catalytic subunit (human telomerase reverse transcriptase (hTERT), telomerase-associated protein 1 (TEP1), heat shock protein 90 kDa (Hsp90), and p23) and an RNA subunit (human telomerase RNA component (hTR)). Telomerase elongates a chromosomal telomere, which is a repeated region of TTAGGG nucleotides.

## OBP-301-BASED ONCOLYTIC VIROTHERAPY

### Preclinical Study of OBP-301

To achieve tumor specificity of virus replication in oncolytic virotherapy, we generated a telomerase-specific replication-competent oncolytic adenovirus, OBP-301, in which the tumor-specific hTERT promoter regulates the

expression of adenoviral *E1A* and *E1B* genes [11] (Figure 2). In normal somatic cells without telomerase activity, OBP-301 cannot replicate due to impairment of hTERT promoter activity. In contrast, OBP-301 can replicate in cancer cells with telomerase activity due to activation of the hTERT promoter and this replication results in the induction of cell lysis and virus spread at 48 to 72 hours after infection (Figure 2). In preclinical experiments, OBP-301 showed an antitumor effect against a variety of human cancer cell lines with telomerase activities [17, 28]. Both epithelial and mesenchymal types of malignant tumor cells were sensitive to OBP-301 infection. Although various types of oncolytic adenoviruses induce cell death associated with autophagy rather than with apoptosis [29], the molecular mechanism underlying the oncolytic adenovirus-mediated autophagic cell death remains unclear. We recently showed that OBP-301-mediated cytopathic activity was significantly associated with induction of autophagy-related cell death, in which adenoviral E1A-dependent activation of transcription factor E2F1 upregulates the expression of microRNA-7, which subsequently downregulates the expression of oncogenic epidermal growth factor receptor (EGFR) [30] (Figure 3). When OBP-301 was intratumorally injected in subcutaneous and orthotopic xenograft tumor models, OBP-301 showed an antitumor effect against both primary tumors [11, 28] and regional lymph node metastasis [31, 32]. These findings suggest that OBP-301 has a broad range of antitumor effects against many kinds of malignant tumor cells with telomerase activity.

To further enhance the antitumor effect of OBP-301-based oncolytic virotherapy, we evaluated the therapeutic potential of OBP-301 in combination with conventional chemotherapy and radiotherapy. Combination therapy with OBP-301 and chemotherapy induces a more profound antitumor effect compared to monotherapy with OBP-301 [33]. OBP-301 synergistically enhanced the antitumor effect of various types of chemotherapeutic agents, such as gemcitabine [34], cisplatin [35-37], paclitaxel [36], and doxorubicin [37]. Regarding the molecular mechanism underlying the OBP-301-mediated enhancement of chemosensitivity, we recently showed that OBP-301 infection results in accumulation of the adenoviral E1A and subsequent E2F1 upregulation, which results in microRNA-29 activation that decreases the expression of anti-apoptotic myeloid cell leukemia 1 (MCL1) protein and subsequently increases chemotherapy-induced apoptotic cell death [37] (Figure 3). The antitumor effect of radiotherapy is also enhanced by OBP-301 infection. Radiotherapy mainly induces DNA double strand-breaks (DSBs) and DNA damage-related cell death in tumor cells. Radiation-induced DSBs can be repaired by accumulation of the Mre11-Rad50-NBS1 (MRN) complex and

MRN complex-mediated activation of ataxiatelangiectasia mutated (ATM) [38] (Figure 4). We showed that OBP-301 infection induces accumulation of the adenoviral E1B protein, which causes degradation of the MRN complex, ATM inactivation, and impairment of radiation-induced DSBs repair [39] (Figure 4). These results suggest that OBP-301-mediated oncolytic virotherapy is a potential antitumor therapy either as a monotherapy or in combination with conventional chemotherapy and radiotherapy.

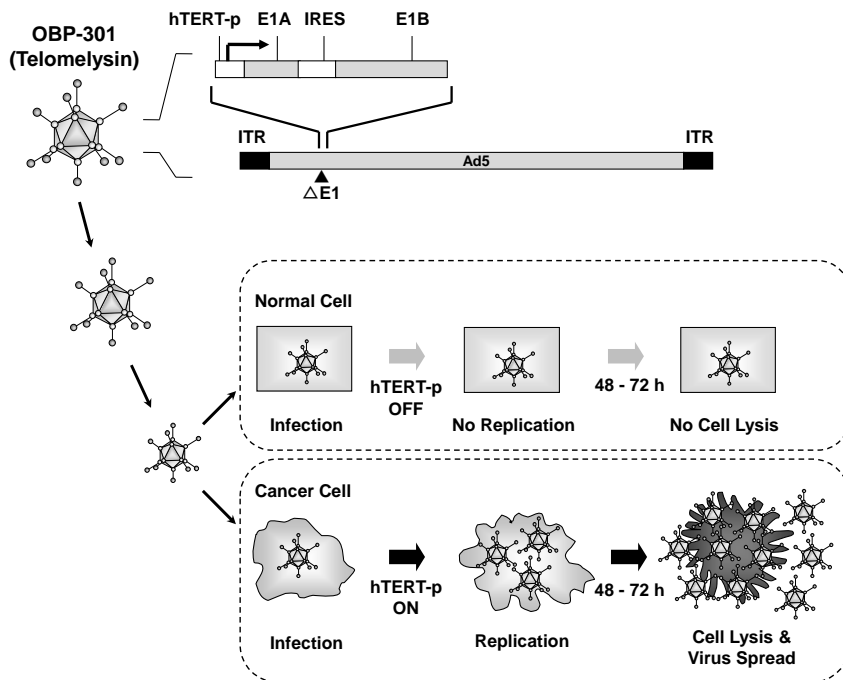


Figure 2. OBP-301-mediated tumor-specific cell death. In OBP-301 (Telomelysin), the human telomerase reverse transcriptase promoter (hTERT-p) drives the expression of *E1A* and *E1B* genes that are linked with an IRES for virus replication. When normal cells without telomerase activity are infected with OBP-301, OBP-301 can enter but cannot replicate within the cells, which switch off the hTERT-p activity. In contrast, when cancer cells with telomerase activity are infected with OBP-301, OBP-301 can enter the cells and can replicate within the cells, which switch on the hTERT-p activity. At 48 to 72 hours after infection, OBP-301 induces cell lysis in cancer cells, but not in normal cells, and causes virus spread into surrounding cancer cells.

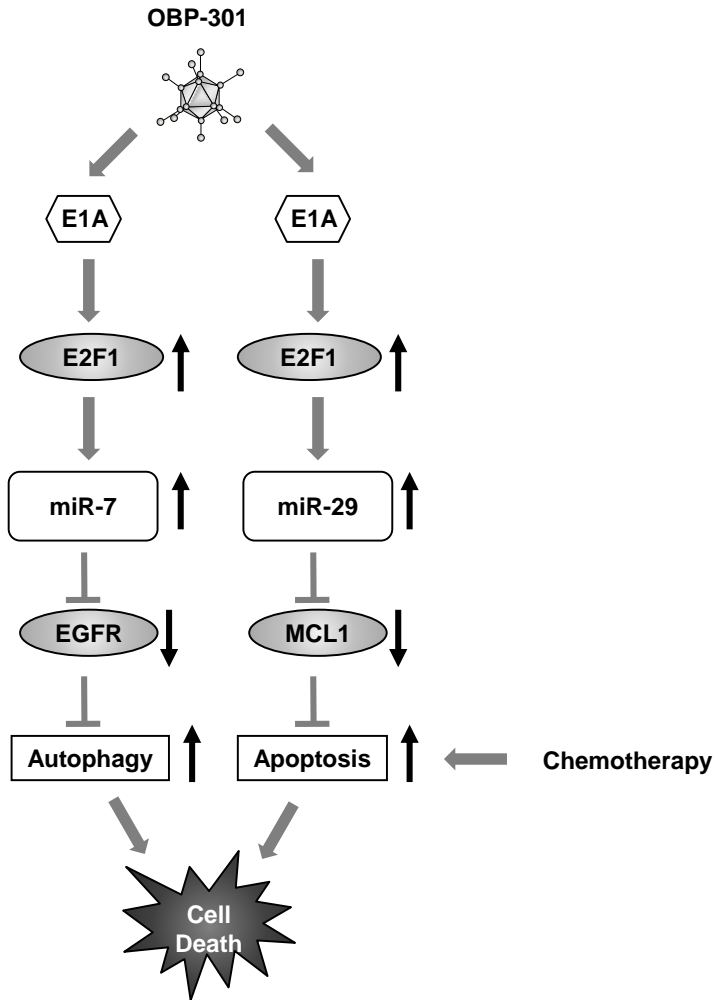


Figure 3. Schematic diagram of OBP-301-mediated tumor-specific cell death in monotherapy or in combination with chemotherapy. The oncolytic adenovirus OBP-301 induces E1A-dependent cell death signaling pathways in association with autophagy and apoptosis through regulation of the E2F1-inducible microRNA network. In monotherapy, OBP-301 induces E1A-mediated E2F1 upregulation and miR-7-mediated epidermal growth factor receptor (EGFR) downregulation, leading to induction of autophagy-related cell death. In combination with chemotherapy, OBP-301 induces E1A-mediated E2F1 upregulation and miR-29-mediated myeloid cell leukemia 1 (MCL1) downregulation, leading to enhancement of chemotherapy-induced apoptosis.

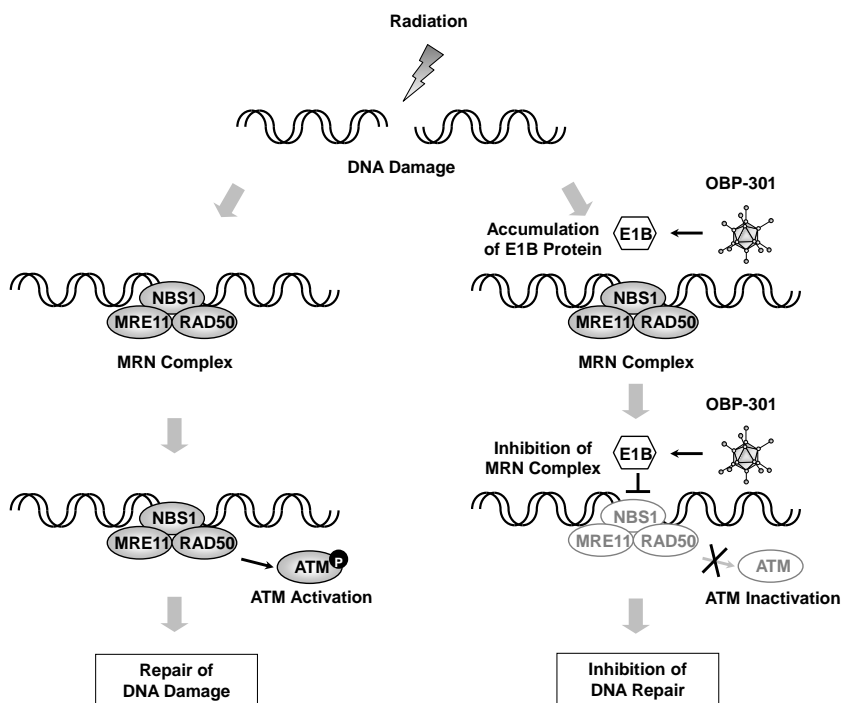


Figure 4. Schematic diagram of the molecular mechanism of OBP-301-mediated DNA repair inhibition in combination with radiation. Radiation causes DNA damage including double stranded breaks in the irradiated cancer cells. In cancer cells not infected with OBP-301, the MRN complex consisting of MRE11, RAD50, and NBS1 binds to the damaged DNA region and induces the activation of ATM phosphorylation, resulting in the repair of DNA damage and the maintenance of cell survival. In contrast, in the OBP-301-infected cancer cells, OBP-301 induces accumulation of the E1B protein, which causes degradation of the MRN complex and inactivation of ATM, and results in the inhibition of DNA damage repair and the induction of cell death.

## Clinical Application of OBP-301

For clinical application of OBP-301, Oncolys BioPharma Inc. was established in March 2004 as an Okayama University-launched bio-venture company. Based on preclinical studies, a phase I clinical trial of OBP-301 was initiated in October 2006 to validate the safety, local response, and pharmacodynamics of OBP-301 as monotherapy in patients with advanced solid tumors in the United States [40]. Sixteen patients with various types of cancer were treated with an intratumoral single injection of OBP-301. This study was

a dose-escalation study, which was designed to enroll cancer patients in 3 cohorts with increasing doses of OBP-301 (cohort 1,  $1 \times 10^{10}$  virus particles (vp); cohort 2,  $1 \times 10^{11}$  vp; cohort 3,  $1 \times 10^{12}$  vp). There were 3 patients/cohort in Cohorts 1 and 2, and 10 patients/cohort in Cohort 3. There was no severe adverse effect related to OBP-301 injection. OBP-301 was well tolerated at all dose levels.

Moreover, based on the radiosensitizing effect of OBP-301 in preclinical studies [39], a phase I/II clinical study of combination therapy with OBP-301 and radiation was initiated in November 2013 to evaluate the safety and feasibility of OBP-301 as combination therapy in Japan (UMIN000010158). This study consisted of 3 cohorts with increasing doses of OBP-301, similar to the phase I clinical trial in the United States. Intratumoral injection of OBP-301 was performed on days 1, 18, and 32 of treatment. Radiation therapy was administered concurrently over 6 weeks to a total of 60 Gy. Six esophageal cancer patients who were not eligible for standard treatments such as surgery and chemotherapy were enrolled in Cohort 1. The Cohort 2 of this study is underway to treat patients with esophageal cancer.

## **OBP-401-BASED SYSTEM FOR TUMOR CELL DETECTION**

### **Preclinical Study of OBP-401**

To detect tumor cells with telomerase activity, we developed a telomerase-specific replication-competent oncolytic adenovirus OBP-401 (TelomeScan), in which a GFP expression cassette was inserted into the E3 region of OBP-301 [21] (Figure 5). OBP-401 tumor-selectively induces GFP expression in a telomerase-dependent manner. Thus, in normal somatic cells without telomerase activity, OBP-401 cannot replicate due to inactivation of the hTERT promoter and GFP expression is not detectable after OBP-401 infection. In contrast, in cancer cells with telomerase activity, OBP-401 can replicate due to activation of the hTERT promoter and GFP expression is detectable at 24 to 48 hours after OBP-401 infection (Figure 5). The GFP-positive tumor cells can be visualized by blue light irradiation under a fluorescence microscope. In preclinical studies, OBP-401 induced GFP expression in a variety of human cancer cell lines with telomerase activities [21, 22]. Both epithelial and mesenchymal types of malignant tumor cells could be detected as GFP-positive cells after OBP-401 infection. When OBP-401 was intratumorally injected in subcutaneous and orthotopic xenograft tumor models, OBP-401 induced GFP



expression in both primary tumors and metastatic tumors at regional lymph nodes or in liver [21, 23, 24]. Moreover, intrapleural or intraperitoneal injection of OBP-401 was also a highly sensitive and useful method for assessment of the dissemination of metastatic tumor cells in mice [25]. These findings suggest that the GFP expression of OBP-401 can be induced by a broad range of malignant tumor cells with telomerase activity.

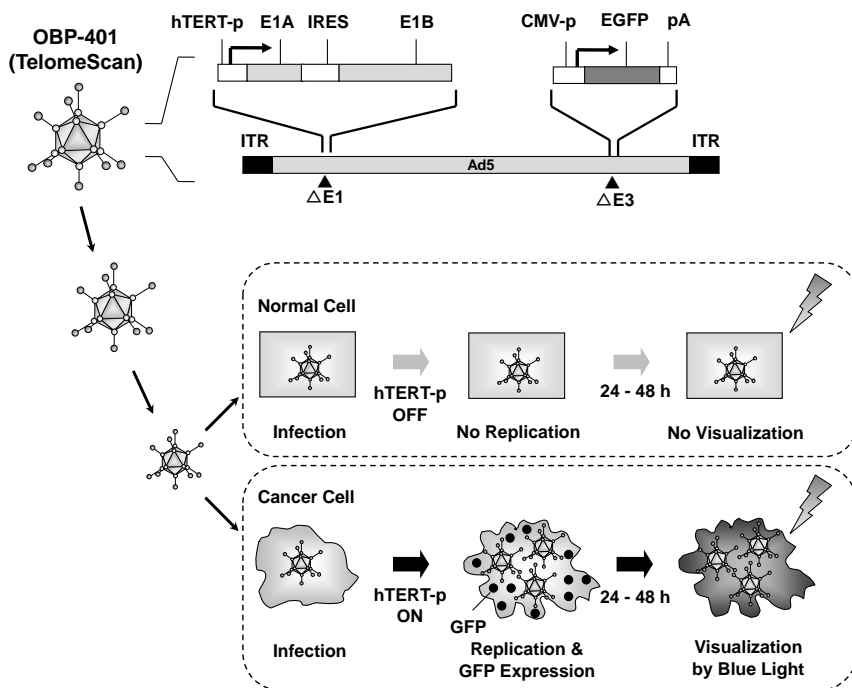


Figure 5. An OBP-401-mediated tumor-specific imaging system. In OBP-401 (TelomeScan), the human telomerase reverse transcriptase promoter (hTERT-p) drives the expression of *E1A* and *E1B* genes that are linked with an IRES for virus replication, as well as the expression of green fluorescence protein (GFP) for tumor imaging. When normal cells without telomerase activity are infected with OBP-401, OBP-401 cannot induce GFP expression in normal cells, which switch off the hTERT-p activity. In contrast, when cancer cells with telomerase activity are infected with OBP-401, the hTERT-p activity is switched on and OBP-401 can induce GFP expression. At 24 to 48 hours after infection, OBP-401 has induced GFP expression in cancer cells, but not in normal cells, and GFP-positive cancer cells can be visualized by blue light irradiation.

## Clinical Application of OBP-401

Recent accumulating evidences have shown that CTC are a predictive biomarker for the assessment of high-risk cancer patients with future distant metastasis [41, 42]. To develop a highly sensitive CTC detection system, we assessed the diagnostic potential of OBP-401 for detecting CTC by using blood samples containing various types of human cancer cell lines [26, 27] (Figure 6).

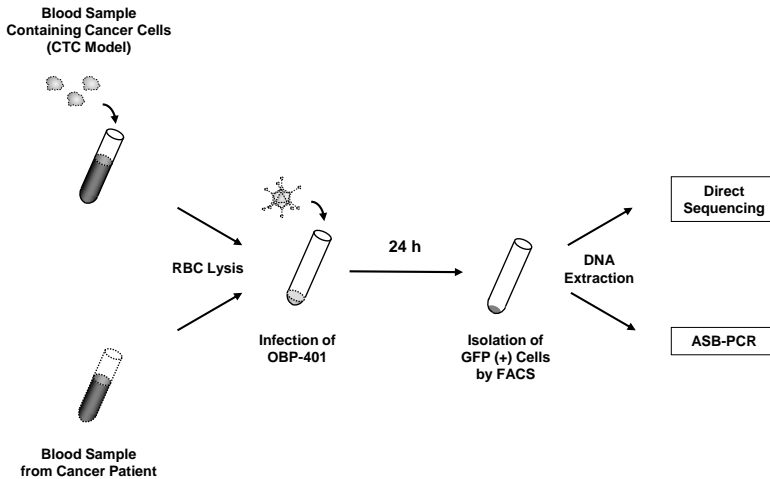


Figure 6. Outline of the OBP-401-mediated system for detection of circulating tumor cells. Blood samples containing human colorectal cancer cell lines with mutations in *KRAS*, *BRAF*, and *KIT* genes are used as models of circulating tumor cells (CTC) for the molecular characterization of CTC. After lysis of the red blood cells (RBC), the blood samples containing cancer cells are infected with OBP-401 for 24 hours. Subsequently, GFP-positive cells are isolated by FACS analysis, and genomic DNA extracted from these GFP-positive cells is subjected to direct sequencing or allele-specific blocker PCR (ASB-PCR) analysis. Additionally, blood samples obtained from patients with *KRAS*- or *BRAF*-mutated colorectal cancers are also analyzed in a similar manner for the assessment of genetic alterations in CTC.

The OBP-401-based CTC detection system was more sensitive for detection of a small number of CTC than quantitative RT-PCR analysis of the *hTERT* gene [26]. The sensitivity of the OBP-401-based CTC detection system was further confirmed using blood samples from cancer patients with various types of cancers, including esophagus [43], stomach [26, 43, 44], colon [26, 27, 43], liver [26, 43], pancreas [43], lung [26, 45, 46], breast [26, 47], cervix [43, 48], endometrium [43, 48], ovary [48], bladder [49], prostate [43], and brain [50].

This evidence showed that the OBP-401-mediated CTC detection system is a broadly useful method for the assessment of cancer patients with premetastatic CTC. Moreover, by combining OBP-401 and FACS analysis, it was possible to isolate the small number of GFP-positive CTC and to analyze the genetic mutation status of the CTC by direct sequencing or by mutation-specific PCR analysis [27] (Figure 6). Although currently the CellSearch system is the only CTC detection system that has been approved by the Food and Drug Administration in the United States [51], that system uses magnetized antibodies that target the epithelial cell adhesion molecule for the enrichment of CTC. It is therefore difficult to detect mesenchymal types of CTC by using the CellSearch system. In contrast, the OBP-401-based CTC detection system can detect both epithelial and mesenchymal types of CTC with telomerase activity. Thus, induction of tumor-specific accumulation of a fluorescent protein by using an oncolytic virus-based gene transfer system would be a useful strategy for the detection of residual tumor cells in a variety of organs and blood.

## CONCLUSION

Oncolytic virotherapy is a promising antitumor strategy for inducing tumor-specific cell death without damaging normal tissues. To generate a genetically bioengineered tumor-specific targeted oncolytic virus, tumor specificity for virus replication or infection is needed. Since telomerase activity is a critical factor for the unlimited proliferation of tumor cells, the telomerase-specific replicative oncolytic adenoviruses, OBP-301 and OBP-401, are useful tools for cancer treatment and diagnosis, respectively. The hTERT promoter-driven oncolytic adenovirus OBP-301 has a broad range of antitumor activity against epithelial and mesenchymal types of malignant tumor cells with telomerase activity in monotherapy or in combination therapy with chemotherapy or radiotherapy. A better understanding of the precise molecular mechanism of the OBP-301-mediated tumor suppression system would provide further novel insights for the improvement of OBP-301-based oncolytic virotherapy. Additionally, the hTERT promoter-driven oncolytic adenovirus OBP-401 is a useful tool for the detection of residual malignant tumor cells in various organs and circulating in blood. In the near future, the OBP-401-based system for capture of tumor cells should improve the assessment of patients with premetastatic advanced cancers.

## ACKNOWLEDGMENTS

This study was supported by grants from the Ministry of Health, Labour and Welfare of Japan and from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

## REFERENCES

- [1] Eager RM, Nemunaitis J. Clinical development directions in oncolytic viral therapy. *Cancer Gene Ther.* 2011;18:305-17.
- [2] Russell SJ, Peng KW, Bell JC. Oncolytic virotherapy. *Nat Biotechnol.* 2012;30:658-70.
- [3] Kirn D, Martuza RL, Zwiebel J. Replication-selective virotherapy for cancer: Biological principles, risk management and future directions. *Nat Med.* 2001;7:781-7.
- [4] Hawkins LK, Lemoine NR, Kirn D. Oncolytic biotherapy: a novel therapeutic platform. *Lancet Oncol.* 2002;3:17-26.
- [5] Chiocca EA. Oncolytic viruses. *Nat Rev Cancer.* 2002;2:938-50.
- [6] Vaha-Koskela MJ, Heikkilä JE, Hinkkanen AE. Oncolytic viruses in cancer therapy. *Cancer Lett.* 2007;254:178-216.
- [7] Wirth T, Zender L, Schulte B, Mundt B, Plentz R, Rudolph KL, et al. A telomerase-dependent conditionally replicating adenovirus for selective treatment of cancer. *Cancer Res.* 2003;63:3181-8.
- [8] Kim E, Kim JH, Shin HY, Lee HS, Sohn JH, Yang JM, et al. Development of a Conditional Replication Competent Adenovirus, Controlled by the Human Telomerase Promoter (hTERT). *Cancer research and treatment: official journal of Korean Cancer Association.* 2003;35:191-206.
- [9] Lanson NA, Jr., Friedlander PL, Schwarzenberger P, Kolls JK, Wang G. Replication of an adenoviral vector controlled by the human telomerase reverse transcriptase promoter causes tumor-selective tumor lysis. *Cancer Res.* 2003;63:7936-41.
- [10] Zou W, Luo C, Zhang Z, Liu J, Gu J, Pei Z, et al. A novel oncolytic adenovirus targeting to telomerase activity in tumor cells with potent. *Oncogene.* 2004;23:457-64.
- [11] Kawashima T, Kagawa S, Kobayashi N, Shirakiya Y, Umeoka T, Teraishi F, et al. Telomerase-specific replication-selective virotherapy for human cancer. *Clin Cancer Res.* 2004;10:285-92.

- [12] Kohno S, Nakagawa K, Hamada K, Harada H, Yamasaki K, Hashimoto K, et al. Midkine promoter-based conditionally replicative adenovirus for malignant glioma therapy. *Oncology reports*. 2004;12:73-8.
- [13] Kubo S, Kawasaki Y, Yamaoka N, Tagawa M, Kasahara N, Terada N, et al. Complete regression of human malignant mesothelioma xenografts following local injection of midkine promoter-driven oncolytic adenovirus. *J Gene Med*. 2010;12:681-92.
- [14] Ono HA, Davydova JG, Adachi Y, Takayama K, Barker SD, Reynolds PN, et al. Promoter-controlled infectivity-enhanced conditionally replicative adenoviral vectors for the treatment of gastric cancer. *Journal of gastroenterology*. 2005;40:31-42.
- [15] Ulasov IV, Tyler MA, Zhu ZB, Han Y, He TC, Lesniak MS. Oncolytic adenoviral vectors which employ the survivin promoter induce glioma oncolysis via a process of beclin-dependent autophagy. *Int J Oncol*. 2009;34:729-42.
- [16] Kyo S, Takakura M, Fujiwara T, Inoue M. Understanding and exploiting hTERT promoter regulation for diagnosis and treatment of human cancers. *Cancer Sci*. 2008;99:1528-38.
- [17] Hashimoto Y, Watanabe Y, Shirakiya Y, Uno F, Kagawa S, Kawamura H, et al. Establishment of biological and pharmacokinetic assays of telomerase-specific replication-selective adenovirus. *Cancer Sci*. 2008;99:385-90.
- [18] Hashimoto Y, Tazawa H, Teraishi F, Kojima T, Watanabe Y, Uno F, et al. The hTERT promoter enhances the antitumor activity of an oncolytic adenovirus under a hypoxic microenvironment. *PLoS One*. 2012;7:e39292.
- [19] Yoon AR, Hong J, Kim SW, Yun CO. Redirecting adenovirus tropism by genetic, chemical, and mechanical modification of the adenovirus surface for cancer gene therapy. *Expert opinion on drug delivery*. 2016;13:843-58.
- [20] Nakashima H, Kaur B, Chiocca EA. Directing systemic oncolytic viral delivery to tumors via carrier cells. *Cytokine and growth factor reviews*. 2010;21:119-26.
- [21] Kishimoto H, Kojima T, Watanabe Y, Kagawa S, Fujiwara T, Uno F, et al. In vivo imaging of lymph node metastasis with telomerase-specific replication-selective adenovirus. *Nat Med*. 2006;12:1213-9.
- [22] Sasaki T, Tazawa H, Hasei J, Osaki S, Kunisada T, Yoshida A, et al. A simple detection system for adenovirus receptor expression using a

- telomerase-specific replication-competent adenovirus. *Gene Ther.* 2013;20:112-8.
- [23] Kurihara Y, Watanabe Y, Onimatsu H, Kojima T, Shiota T, Hatori M, et al. Telomerase-specific virotheranostics for human head and neck cancer. *Clin Cancer Res.* 2009;15:2335-43.
- [24] Kishimoto H, Urata Y, Tanaka N, Fujiwara T, Hoffman RM. Selective metastatic tumor labeling with green fluorescent protein and killing by systemic administration of telomerase-dependent adenoviruses. *Mol Cancer Ther.* 2009;8:3001-8.
- [25] Kishimoto H, Zhao M, Hayashi K, Urata Y, Tanaka N, Fujiwara T, et al. In vivo internal tumor illumination by telomerase-dependent adenoviral GFP for precise surgical navigation. *Proc Natl Acad Sci U S A.* 2009;106:14514-7.
- [26] Kojima T, Hashimoto Y, Watanabe Y, Kagawa S, Uno F, Kuroda S, et al. A simple biological imaging system for detecting viable human circulating tumor cells. *J Clin Invest.* 2009;119:3172-81.
- [27] Shigeyasu K, Tazawa H, Hashimoto Y, Mori Y, Nishizaki M, Kishimoto H, et al. Fluorescence virus-guided capturing system of human colorectal circulating tumour cells for non-invasive companion diagnostics. *Gut.* 2015;64:627-35.
- [28] Sasaki T, Tazawa H, Hasei J, Kunisada T, Yoshida A, Hashimoto Y, et al. Preclinical evaluation of telomerase-specific oncolytic virotherapy for human bone and soft tissue sarcomas. *Clin Cancer Res.* 2011;17:1828-38.
- [29] Tazawa H, Kagawa S, Fujiwara T. Oncolytic adenovirus-induced autophagy: tumor-suppressive effect and molecular basis. *Acta medica Okayama.* 2013;67:333-42.
- [30] Tazawa H, Yano S, Yoshida R, Yamasaki Y, Sasaki T, Hashimoto Y, et al. Genetically engineered oncolytic adenovirus induces autophagic cell death through an E2F1-microRNA-7-epidermal growth factor receptor axis. *Int J Cancer.* 2012;131:2939-50.
- [31] Kojima T, Watanabe Y, Hashimoto Y, Kuroda S, Yamasaki Y, Yano S, et al. In vivo biological purging for lymph node metastasis of human colorectal cancer by telomerase-specific oncolytic virotherapy. *Ann Surg.* 2010;251:1079-86.
- [32] Kikuchi S, Kishimoto H, Tazawa H, Hashimoto Y, Kuroda S, Nishizaki M, et al. Biological ablation of sentinel lymph node metastasis in submucosally invaded early gastrointestinal cancer. *Mol Ther.* 2015;23:501-9.

- [33] Fujiwara T, Kagawa S, Tazawa H. Synergistic interaction of telomerase-specific oncolytic virotherapy and chemotherapeutic agents for human cancer. *Curr Pharm Biotechnol*. 2012;13:1809-16.
- [34] Liu D, Kojima T, Ouchi M, Kuroda S, Watanabe Y, Hashimoto Y, et al. Preclinical evaluation of synergistic effect of telomerase-specific oncolytic virotherapy and gemcitabine for human lung cancer. *Mol Cancer Ther*. 2009;8:980-7.
- [35] Takakura M, Nakamura M, Kyo S, Hashimoto M, Mori N, Ikoma T, et al. Intraperitoneal administration of telomerase-specific oncolytic adenovirus sensitizes ovarian cancer cells to cisplatin and affects survival in a xenograft model with peritoneal dissemination. *Cancer Gene Ther*. 2010;17:11-9.
- [36] Yano S, Tazawa H, Hashimoto Y, Shirakawa Y, Kuroda S, Nishizaki M, et al. A genetically engineered oncolytic adenovirus decoys and lethally traps quiescent cancer stem-like cells in S/G2/M phases. *Clin Cancer Res*. 2013;19:6495-505.
- [37] Osaki S, Tazawa H, Hasei J, Yamakawa Y, Omori T, Sugiu K, et al. Ablation of MCL1 expression by virally induced microRNA-29 reverses chemoresistance in human osteosarcomas. *Scientific reports*. 2016;6:28953.
- [38] Kuroda S, Urata Y, Fujiwara T. Ataxia-telangiectasia mutated and the Mre11-Rad50-NBS1 complex: promising targets for radiosensitization. *Acta medica Okayama*. 2012;66:83-92.
- [39] Kuroda S, Fujiwara T, Shirakawa Y, Yamasaki Y, Yano S, Uno F, et al. Telomerase-dependent oncolytic adenovirus sensitizes human cancer cells to ionizing radiation via inhibition of DNA repair machinery. *Cancer Res*. 2010;70:9339-48.
- [40] Nemunaitis J, Tong AW, Nemunaitis M, Senzer N, Phadke AP, Bedell C, et al. A phase I study of telomerase-specific replication competent oncolytic adenovirus (telomelysin) for various solid tumors. *Mol Ther*. 2010;18:429-34.
- [41] Joosse SA, Gorges TM, Pantel K. Biology, detection, and clinical implications of circulating tumor cells. *EMBO molecular medicine*. 2015;7:1-11.
- [42] Massague J, Obenauf AC. Metastatic colonization by circulating tumour cells. *Nature*. 2016;529:298-306.
- [43] Yabusaki M, Sato J, Kohyama A, Kojima T, Nobuoka D, Yoshikawa T, et al. Detection and preliminary evaluation of circulating tumor cells in

- the peripheral blood of patients with eight types of cancer using a telomerase-specific adenovirus. *Oncology reports*. 2014;32:1772-8.
- [44] Ito H, Inoue H, Sando N, Kimura S, Gohda K, Sato J, et al. Prognostic impact of detecting viable circulating tumour cells in gastric cancer patients using a telomerase-specific viral agent: a prospective study. *BMC cancer*. 2012;12:346.
- [45] Dorsey JF, Kao GD, MacArthur KM, Ju M, Steinmetz D, Wileyto EP, et al. Tracking viable circulating tumor cells (CTCs) in the peripheral blood of non-small cell lung cancer (NSCLC) patients undergoing definitive radiation therapy: pilot study results. *Cancer*. 2015;121:139-49.
- [46] Igawa S, Gohda K, Fukui T, Ryuge S, Otani S, Masago A, et al. Circulating tumor cells as a prognostic factor in patients with small cell lung cancer. *Oncology letters*. 2014;7:1469-73.
- [47] Kim SJ, Masago A, Tamaki Y, Akazawa K, Tsukamoto F, Sato J, et al. A novel approach using telomerase-specific replication-selective adenovirus for detection of circulating tumor cells in breast cancer patients. *Breast Cancer Res Treat*. 2011;128:765-73.
- [48] Takakura M, Kyo S, Nakamura M, Maida Y, Mizumoto Y, Bono Y, et al. Circulating tumour cells detected by a novel adenovirus-mediated system may be a potent therapeutic marker in gynaecological cancers. *Br J Cancer*. 2012;107:448-54.
- [49] Ju M, Kao GD, Steinmetz D, Chandrasekaran S, Keefe SM, Guzzo TJ, et al. Application of a telomerase-based circulating tumor cell (CTC) assay in bladder cancer patients receiving postoperative radiation therapy: a case study. *Cancer biology and therapy*. 2014;15:683-7.
- [50] Macarthur KM, Kao GD, Chandrasekaran S, Alonso-Basanta M, Chapman C, Lustig RA, et al. Detection of brain tumor cells in the peripheral blood by a telomerase promoter-based assay. *Cancer Res*. 2014;74:2152-9.
- [51] Miller MC, Doyle GV, Terstappen LW. Significance of Circulating Tumor Cells Detected by the CellSearch System in Patients with Metastatic Breast Colorectal and Prostate Cancer. *Journal of oncology*. 2010;2010:617421.





## *Chapter 5*

# **EVALUATION OF THE SWELLING AND DIFFUSIONAL BEHAVIOR OF GUAR GUM FOR THE CONTROLLED RELEASE OF BIOACTIVE AGENTS**

***John Rojas\* and Yhors Ciro***

Department of Pharmacy, University of Antioquia,  
Medellín, Columbia

## **ABSTRACT**

Guar gum is a hydrophilic polymer that has gained attention for the fabrication of matrices for controlled solute release due to its gelling nature and ability to entrap the solute within the gel. It is highly soluble in water, stable over a wide range of temperature, acidic, alkaline and enzymatic conditions. Matrix systems are made of polymeric materials that are swellable in the presence of biological fluids. The powdered solute was distributed uniformly in a matrix of guar gum and directly compressed to form a tablet using a single punch tablet press equipped with a flat-faced tooling (13 mm diameter). A guar gum matrix tablet is cost effective and have broad FDA acceptance.

The swelling characteristics of guar gum matrix tablets were studied using three solutes having different solubilities. Thus, methylene blue,

---

\* Corresponding author: John Rojas. Cll 67 # 53-108, office 1-157. Medellín, Tel.: 574 219 5472.  
Email address: jrojasca@gmail.com.

caffeine and salicylic acid were used as soluble, highly soluble and acidic compounds. Swelling was assessed by measuring the axial and radial expansion of matrix tablets following exposure to distilled water, acidic and brine media. The mechanisms of solute release and matrix swelling rate were calculated from the dissolution and swelling experiments, respectively. Matrix swelling was related to the intake of a large amount of water and formation of a viscoelastic mass. A rapid absorption of water took place through permeation and capillary action. Solute release kinetics included mainly relaxation rather than diffusion transport. Solute release was also influenced by the presence of ions and pH of the media. The mechanism of solute release from this polymeric matrix mostly conforms to non-Fickian (anomalous) transport. Thus, swelling played an important role to obtain complete solute release within 24 h. Further, the ionic strength of the liquid had a strong effect on the sorption properties of the matrix.

Solute release from guar gum matrices was preceded mainly by a combination of swelling and diffusion mechanisms depending on the chemical nature of the solute employed. Kinetics of solute release from these matrices depended mainly on the synchronization of polymer hydration at the moving rubbery/glassy front within the matrix and the rate of solute diffusion.

**Keywords:** guar gum, controlled release, bioactive agents, swelling behavior

## INTRODUCTION

In the last decade, modified release systems of solutes have become popular because they provide advantages over the traditional immediate release systems. These advantages include a reduced administration frequency, better therapeutic control, and reduced side effects and interactions [1]. The development of a modified release dosage form can be achieved through matrix devices. These consist of homogeneous mixtures of a solute with a polymer that controls the release rate. Subsequently, the solute is released by diffusion, swelling or erosion transport mechanisms. Further, matrix devices could be classified as inert, hydrophilic and lipophilic systems depending on their physical and chemical characteristics. Inert matrices are also referred as plastic matrices and are able to form a solid porous network upon contact with the gastrointestinal tract. In this case, solute release occurs by diffusion through the pores of the matrix and an influx of the media through the porous network system occurs by capillarity simultaneously. Once the solute is dissolved it diffuses through the

solvent-filled capillaries. Typically, inert matrices are able to release their solute load and remain intact even after interacting with acidic media [2]. On the other hand, lipid matrices are composed by a fatty substance which is responsible for controlling the solute release rate. The most common fatty materials employed include glycerides, fatty acids, and fatty acid esters of low molecular weight. These are naturally occurring materials which are well tolerated physiologically. Usually, the preparation of these matrices includes melting of the component mixture followed by spray-drying and tableting. The major issue with these matrices is the potential fluctuation of the lipase content among individuals which results in a variation of the solute release rates. The third type of matrix devices is represented by the hydrophilic matrix tablets, which are also known as hydrogel matrices or swellable matrix tablets. These materials function as a swelling controlled release systems. These matrices hydrate and swell upon contact with water resulting in a controlled release of the solute. Typically, swelling polymers such as cellulose ethers, acrylic polymers such as Carbopol®, locust bean gum, alginic acid, guar gum and carrageenan could function as hydrophilic matrix tablets [3, 4].

Guar gum is a polysaccharide obtained from the endosperm of *Cyamopsis tetragonolobus* (Linné) and it is composed of galactan and mannan units combined through glycosidic linkages. The hydration rate and stability are constant in a pH ranging from 4 to 10 due to its non-ionic and uncharged character [5]. Upon contact with water molecules, the galactose side chains attached to mannose backbone interact with the surrounding water molecules resulting into inter-molecular chain entanglement and gel formation. Further, it is resistant to dissociation in acidic pH, and it is susceptible to enzymatic and microbial degradation in the large intestine becoming a suitable hydrophilic matrix tablet for controlled delivery of solutes [6].

## SOLUTE RELEASE CHARACTERISTICS

In this chapter, the dissolution profiles of several solutes named as caffeine, methylene blue and salicylic acid from guar gum cylindrical matrices were studied. Physical mixtures of guar gum and the respective solute (~100 mg of salicylic acid, 50 mg of methylene blue, and 200g of caffeine) were prepared and compressed in a single punch tablet press at ~120 MPa and a dwelling time of 1s. These matrix tablets were then submitted to a dissolution test using a type 2 dissolution apparatus at 37°C and 50 rpm for 24h. The possible solvent effect on dissolution profiles were studied on 0.1N HCl, 2.5M NaCl and distilled

water. The release mechanism was evaluated by fitting the release data to the Korschmeyer-Peppas and Peppas-Sahlin models which are suitable for amorphous swellable systems [7–9]:

$$F=kt^n \quad (1)$$

$$F=k_d t^{0.45} + k_r t^{0.89} \quad (2)$$

Where,  $F$  is the fraction of solute released at time  $t$ ,  $k$  is a kinetic constant measuring the velocity of solute release and incorporates characteristics of the macromolecular network system and the solute. Equation 2 is used to determine the relaxational or diffusional contribution on solute transport. The first term of the equation is related to the Fickian (diffusional), whereas the second term is associated to the relaxational (or erosional) contributions to solute release.  $k_d$  and  $k_r$  are the rate constants of the Fickian and the relaxational (erosional) contributions, respectively. Further,  $n$  is the exponent that depends on the release mechanism and the shape of the matrix tested. For instance, in cylindrical matrixes a Fickian diffusion is defined by  $n$  equal to 0.45 and it is driven by a chemical potential gradient. This mechanism occurs when water transport in the polymer is controlled by a concentration gradient. In this case, upon water contact the polymer adopts an equilibrium state almost immediately. Further, this mechanism happens in nonswelling systems, or when the relaxation time of the polymer is much shorter than the diffusion time for water transport. On the other hand, case II transport occurs when the polymer relaxation is the rate limiting step to water transport (relaxation or swelling controlled systems) and is expressed by an  $n$  value of 0.89. In this case, a constant release rate (zero order) is observed when water penetration is much slower than solute diffusion in the swollen gel. Further, an erosion controlled system could also lead to a case II transport [10]. In contrast, a nonFickian or anomalous transport occurs as a result of contributions from diffusion and polymer erosion altogether, and it is expressed by  $n$  values from 0.45 to 0.89. In this case, the water uptake mechanism leads to transport behavior intermediate to Fickian and case II transport [11].

Values of the various corresponding parameters, determined using a nonlinear least squares fitting method, are listed in Table 1. Fickian diffusion appears to be the controlling release mechanism for caffeine in acidic media ( $n$  value of 0.45), whereas nonFickian or anomalous transport prevailed for all compounds ( $0.45 < n < 0.89$ ). On the other hand, methylene blue in brine media exhibited a super case II mechanism where the release of the solute depended

mainly on the swelling or relaxation behavior of guar gum and as a result, it showed the poorest releasing characteristics (Figure 1). Further, the rate constants ( $k$  values) of each solute depended on the solvent used. In this scenario, solutes dissolved faster in distilled water, or acidic media than brine media. Therefore, the presence of sodium chloride altered the intermolecular interactions of guar gum by changing the charge density and chain conformation. This finding is explained by the competition of brine solution with guar gum molecules for the surrounding water molecules resulting in a delay of hydration and hence, the swelling rate of the matrixes.

The increased solubility of caffeine resulted in higher release rate. The larger concentration gradient through the gel layer (although the thickness increased slightly with increasing solubility) accounts for this outcome. Further, matrix erosion becomes the rate limiting factor when caffeine release is conducted in acidic media leading to a Fickian mechanism [12].

Guar gum matrices showed a minimal initial burst of solute, due to the release from the surface and the lag time required for the advance of an operative gel layer capable of controlling water diffusion and solute release. This was predominantly apparent in the case of soluble compounds such as caffeine and salicylic acid releasing from 10 to 20% within 1h independent of the release media. Conversely, the release of methylene blue was highly affected by the presence of ions ( $\text{Na}^+$  and  $\text{Cl}^-$ ) in the media. Therefore, this media also reduced the amount of solute released within 24h to 73%, 50% and 17% for caffeine, salicylic acid and methylene blue, respectively.

**Table 1. Fitting parameters of the solutes according to the Korshmeier-Peppas and Peppas-Sahlin models**

Solute	Solvent	Korschmeier-Peppas model			Peppas-Sahlin model		
		$k$	$n$	$r^2$	$k_d$	$k_r$	$r^2$
Caffeine	Distilled water	0.163	0.55	0.9900	0.158	0.017	0.9846
	2.5M NaCl	0.093	0.66	0.9973	0.077	0.026	0.9951
	0.1M HCl	0.273	0.45	0.9678	0.2931	-0.0073	0.9671
Methylene blue	Distilled water	0.217	0.43	0.9725	0.2338	-0.0094	0.9977
	2.5M NaCl	0.004	1.19	0.9881	0.001	-0.008	0.9801
	0.1M HCl	0.133	0.59	0.9976	0.123	0.022	0.9941
Salicylic acid	Distilled water	0.110	0.64	0.9992	0.092	0.028	0.9967
	2.5M NaCl	0.059	0.67	0.9950	0.024	0.020	0.9961
	0.1M HCl	0.056	0.80	0.9930	0.028	0.035	0.9958

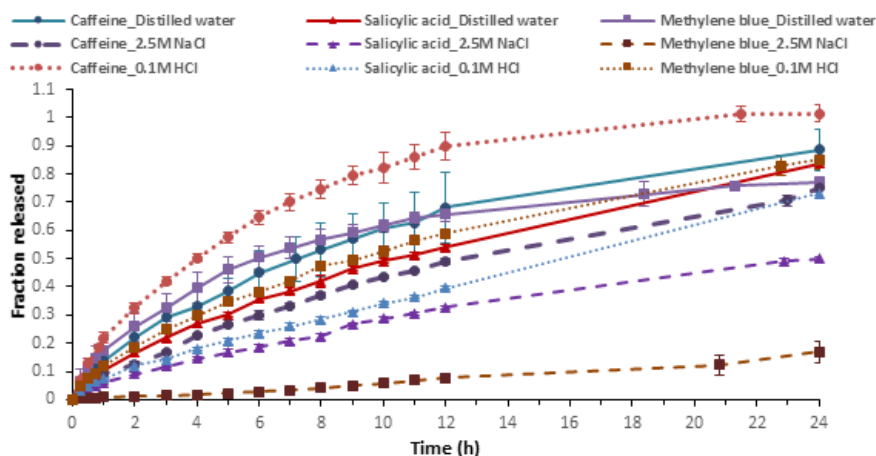


Figure 1. Solute release pattern from guar gum matrixes in different media or solute release curves from swellable guar gum matrixes.

According to equation 2, in a hydrophilic matrix there are two competing mechanisms involved in the solute release: Fickian diffusional release and relaxational release. The relative contribution of each component to the total release is mainly dependent on the properties of a given solute. For instance, the release of a sparingly soluble solute (i.e., salicylic acid) from guar gum matrixes involves the concurrent absorption of water and desorption of solute through a swelling controlled diffusion mechanism. As water penetrates into a guar gum matrix, the polymer swells and its glass transition temperature is lowered. At the same time, the dissolved solute diffuses through this swollen rubbery region into the external releasing solvent. This type of water transport via water diffusion and swelling normally does not lead to a solute Fickian diffusion transport [13].

In order to analyze the contribution to solute release of polymer relaxation (or erosion) and solute diffusion (Fickian) in the gel layer, solute release was described in terms of a relaxational and diffusional (R/F) ratio (Figure 2). This ratio increased with time indicating a profound nonFickian contribution. In other words, a substantial swelling-controlled release could operate, especially in acidic media. Thus, a macromolecular relaxation is most likely the mechanism complementary to Fickian diffusion. For instance, materials such as salicylic acid and methylene blue in brine media exhibited the largest ratio, and the lowest fraction released at the same time. On the contrary, caffeine released in distilled water presented the lowest ratio and hence, the largest fraction released. When inspecting more profoundly the the fraction of solute released, caffeine

showed the most striking features, especially in distilled water and acidic media. This outcome was attributed to the high water solubility of caffeine which has a pKa of 11.1 resulting in a high solubility and hence, dissolution in acidic media. Conversely, salicylic acid exhibited the lowest water solubility and having a pKa of 2.97 presented the lowest release rates in all three media, especially when exposed to a brine environment. Interestingly, methylene blue having a pKa of 3.8 showed an intermediate solubility between caffeine and salicylic acid and formed a complex in presence of sodium chloride as reflected by the gradual formation of a precipitate on the outer surface of the matrix tablet. This result is not surprising since methylene blue has the ability of chelating ionic compounds such as sodium chloride and as a result, a layer of precipitated complex is observed on the surface of the matrix tablet.

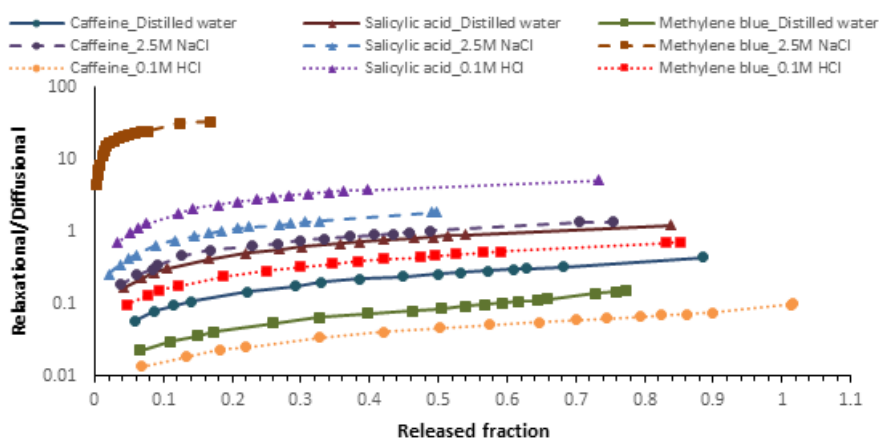


Figure 2. Ratio between relaxational and diffusional contribution to solute release vs. fraction release.

The release area was defined as the area available for solute release. Figure 3 depicts the plot of the fractional release of the solute against the released area. A careful inspection of this plot—illustrates two linear regions. The first one occurs as a result of the formation of an external gel layer upon contact with water. In this segment the water penetration rate was low, continuous, and responsible for a trivial solute release (<20%). Further, in the first hour of the test differences in solute release were not pronounced because of the small matrix volume (gel) exposed to the dissolution medium. The capability to form rapidly a continuous gelified barrier was also related to the hydrophilicity of guar gum in the dissolution medium. Therefore, in the first segment the fraction



of solute released varied in the order: 0.1 N HCl > distilled water > 2.5 N NaCl. This can be ascribed to whether or not guar gum could rapidly form a continuous gel layer on the surface and in the matrix core. Further, solute release differed markedly during the late time points, especially in acidic media for caffeine and methylene blue. Thus, after 22h a retractive deviation from linearity occurred, suggesting that a partial erosion of the guar gum matrix tablet occurred. These results indicate that in acidic media guar gum is more prone to chain disentanglement, and thus to erosion than distilled water. Further, the plot of the fraction release vs. release area shows slight area retraction in the late time points in the acidic medium indicating an erosive matrix behavior which also contributed to solute release. The solute itself increased the solute concentration in the gel (especially for highly soluble compounds such as caffeine) leading to the largest release. A high electrolyte concentration also hindered gel layer formation and consequently solute release.

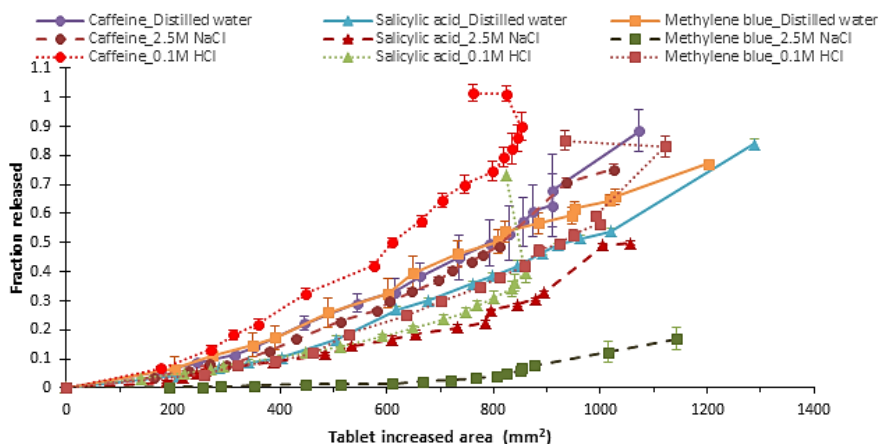


Figure 3. Fraction of solute release vs. release area from guar gum matrices.

## SWELLING MECHANISM ANALYSIS

The degree of swelling was expressed as the normalized volume increase with respect to the initial nonswelled cylindrical matrix. Upon contact of the guar gum matrix with water, a fast initial inward and outward movement of the swelling and erosion fronts was observed. Therefore, the overall matrix growth was ascribed to water uptake. The plot of the gel or swelling volume as a

function of the square root of time is depicted in Figure 4. A predominant circular swelling was observed as a result of growth of ungelled matrix core in a radial direction. Therefore, the swelling volume was described as dependent on the square root of time indicating a Fickian water diffusion for dynamic swelling and thus, the swelling front (the glassy to rubbery state transition) moves more quickly than the lethargic eroding front. However, in most samples after 19h of the experimental run a deviation from Fickian conditions occurred, probably indicating an erosive phenomenon [14].

A slow swelling increase was observed in the first hour for all materials. Subsequently, the gel layer grew at a larger rate exhibiting an almost linear pattern up to ~19h. The results were explained hypothetically in terms of rate of advancement of the swelling front into matrix and diffusion front layer. Further, in most cases swelling of the matrix predominated over the erosion phenomena of guar gum.

Typically, in the first hour of the test water uptake occurred in a 3D pattern where matrix expansion arises in the radial and axial directions simultaneously. In the same period of time, these vectorial expansions were comparable as seen by their almost superimposed slopes. In this case, these matrices had a density larger than that of the release media and remained mostly at the bottom of the dissolution vessel. In fact, in the first hour of the test guar gum matrices swelled mostly in the axial direction, and then the ratio of axial and radial swelling remained virtually constant due to a restriction to swelling on the axial side owing to the glassy core structure.

Further, the ratio of the radial to the axial matrix expansion upon water uptake decreased from 4 to 2-3 in the first hour and then it remained fairly constant between 2 and 3, independent of the drug solubility and release media employed. This is indicative of the prevalent swelling or relaxation characteristics of guar gum on solute release.

Subsequently, after 1-1.5h the rate of axial matrix expansion decreased and become almost constant, whereas the rate of radial expansion remained large and unchanged. As a result, two different linear segments can be appreciated in the plot of swelling volume as a function of the square root of time (Figure 4). The critical time point between these two segments is an indication of the instant when the tablet matrix reaches a critical volume leading to flotation in the release media. At this point, the density of the matrix was equivalent to the density of the aqueous media (~0.99, 1.09 and 1.05 g/cm<sup>3</sup> for distilled water, 2.5M NaCl and 0.1N HCl, respectively). Therefore, the matrix remained floating from 1 to 24h as long as the volume and release area continued rising.

This offer a great advantage to guar gum over other amorphous polymers since it exhibits virtually no erosion or dissolution in neutral or saline media, preserving the integrity of the release gelling structure.

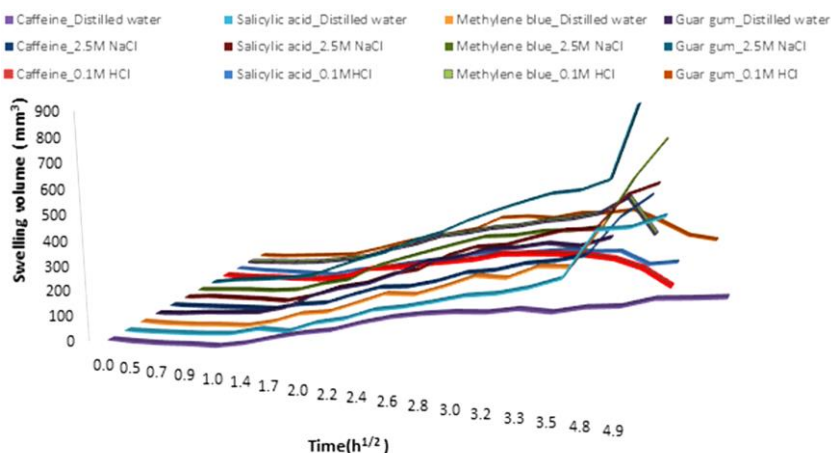


Figure 4. Swelling volume of guar gum matrix tablets as a function of time during solute release.

A swellable guar gum matrix tablet was characterized by a major change of swelling area (Figure 5). The increase of the releasing area during swelling is used to measure the matrix expansion. In fact the amount of solute released was plotted vs. the corresponding swelling area rate resulting in a linear relationship. In general, in guar gum the swelling area increased with time and it was responsible for controlling solute release since polymer erosion was negligible.

Solubility of the solute may have an effect on solute release since the gel layer thickness did not remained constant. This result was seen in matrices containing a solute exhibiting different solubility from swellable cylinders. It is believed that the gel layer thickness increases in value with the solubility of solute due to osmotic effect. At early times, as a result of speedy swelling, the tablet expanded outwardly pulling the diffusion front. Solute solubility was capable of altering water penetration, swelling, and hence solute release [15].

After a sample is swollen, some macromolecular entanglements are broken to yield a different polymer structure and hence a swelling kinetics. A matrix placed in the medium will swell and rearrange to accommodate the solvent. The rate of solvent uptake and compatibility of guar gum with a particular solvent

leads to stresses occurring between the rubbery and glassy areas of the swelled guar gum.

**Table 2. Variation of swelling rates and solute dissolution rates**

Solute, polymer or solvent	Swelling rate (mm <sup>3</sup> /h)					Solute dissolution rate (mg/h)			
	Critical water volume (mm <sup>3</sup> )	k <sub>1</sub>	r <sup>2</sup>	k <sub>2</sub>	r <sup>2</sup>	k <sub>1</sub>	r <sup>2</sup>	k <sub>2</sub>	r <sup>2</sup>
Guar gum (2.5 M NaCl)	74.4	70.6	0.9277	260.7	0.9918	N.A	N.A	N.A	N.A.
Guar gum (0.1N HCl)	49.0	46.7	0.8555	140.9	0.9885	N.A	N.A	N.A	N.A.
Guar gum (distilled water)	40.1	55.1	0.9976	40.2	0.9861	N.A	N.A	N.A	N.A.
Distilled water	34.7	32.9	0.8313	148.6	0.9835	14.5	0.8053	0.36	0.5759
0.1 M HCl	45.1	32.0	0.8382	117.2	0.9931	14.1	0.7013	0.35	0.5304
2.5 M NaCl	38.1	37.2	0.9352	179.6	0.9885	9.5	0.6128	0.06	0.3982
Methylene blue	46.1	43.0	0.8476	159.4	0.9902	5.4	0.5690	0.09	0.7779
Caffeine	30.7	29.7	0.8616	132.3	0.9889	25.2	0.7329	0.57	0.4392
Salicylic acid	41.2	29.1	0.8955	153.7	0.9860	7.4	0.8175	0.13	0.2873

The intrinsic dissolution and flux of caffeine, salicylic acid and methylene blue were 5.7, 0.5 and 4.8 mg/min, and 4.3, 0.4 and 3.6 mg/cm<sup>2</sup>min, respectively. Since salicylic acid is insoluble in water at pH below 3, solute release from the matrices is pH-dependent. At acid pH and in presence of ions, at the tablet surface salicylic acid converts to insoluble form and the gel layer becomes more prompt to erosion which is critical for controlling solute release. Salicylic acid dissolved slowly in the gel layer and gradually spread into the adjacent aqueous environment. Additionally, highly soluble solutes such as caffeine did not show a release rate increased since the erosion front was not significantly affected. Further, the solute release rates were inversely related to the gel layer thickness (swelling volume).

Initially, in the first hour swelling remains fairly constant for materials in contact with distilled water and 0.1M HCl. However, it became larger in acidic media followed by distilled water and 2.5 M NaCl (Table 2). Interestingly, the brine media promoted the increase of the critical water uptake volume in the 1st hour. On the other hand, dissolution rates in the first hour were high for caffeine

and slower in brine media. Thereafter, it was reduced to  $<0.6$  mg/h and remained constant up  $\sim 19$ h. Independent of the media, methylene blue showed the largest swelling constant and caffeine the lowest critical swelling. Subsequently, swelling remained constant for methylene blue and salicylic acid and decreased for caffeine.

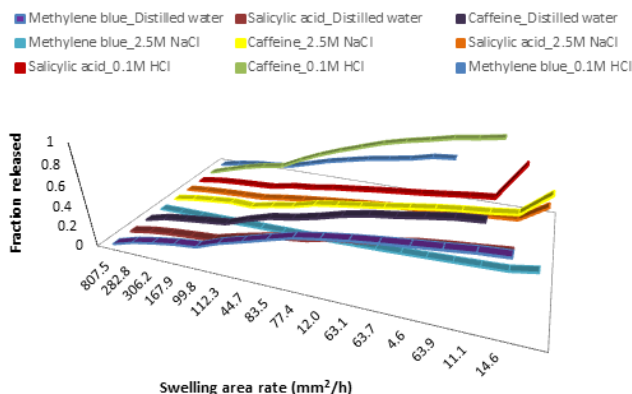


Figure 5. Variation of fraction released fraction as a function swelling area rate in guar gum matrices.

## MOVABLE FRONTS IN GUAR GUM MATRICES

Guar gum matrices exhibited mainly two fronts interacting simultaneously with upcoming water molecules and solutes present inside the matrix integrity (Figure 6). The first one is the swelling front, which is defined as the boundary between the still glassy guar gum and the rubbery segment. On the other hand, the erosion front is the boundary between the matrix tablet and the aqueous solvent. The swelling front movement was related with the water uptake rate, whereas the erosion front is related with the solute dissolution rate and the matrix erosion rate. Kinetics of solute release from guar gum matrix tablets is controlled by the dynamics of these two front movements and hence, it is a function of the solute gel layer. Conversely, none of the solutes showed any zero order release since the gel layer thickness did not remain constant. This happens only in erodible polymers where the two fronts move in a synchronized way keeping constant the releasing area. Further, these findings were corroborated by the absence of “n” release constants equal to 0.89. In guar gum matrices a

truly diffusion front was not visually identified. This front usually occurs in the interface between the undissolved solute and the dissolved solute in the gel segment. Further, solute release rates decreased with increasing of time due to the absence of synchronization of the moving fronts [16].

In general, guar gum matrices were activated by the aqueous media, and solute release control depended on the interactions between guar gum, water, and solute. Water penetration into the matrix was the first step leading to guar gum swelling and solute dissolution. The presence of water decreases the glassy rubbery temperature, giving rise to the transformation of a glassy polymer in a rubbery phase (gel layer). The enhanced mobility of the guar gum chains favored the transport of dissolved solute. Further, the guar gum relaxation phenomena determined the swelling or volume increase ability of the matrix upon contact with water [17].

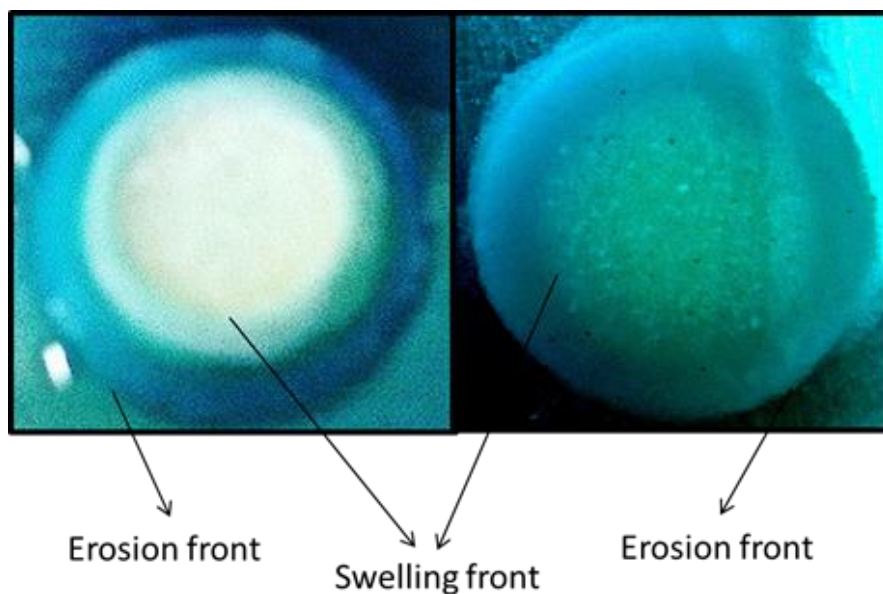


Figure 6. Top of a swellable matrix tablet upon water contact showing the movable fronts.

Depending on the guar gum characteristics, the polymer amount in the rubbery phase at the surface of the matrix could reach the disentanglement concentration forming a gel layer which increases in thickness. Therefore, the gel layer thickness depends on the relative contributions of water penetration,

chain disentanglement, and mass (guar gum and solute) transfer in water. At the beginning, water penetration is more rapid than chain disentanglement and a quick buildup of gel layer volume takes place. Subsequently, water penetrates slowly in the axial direction, due to the increase of the diffusional distance; but the radial expansion remained large due to the absence of—polymer disentanglement rates [18]. Thus, the gel layer thickness dynamics in guar gum matrix tablets was able to prevent matrix disintegration and controlled additional water penetration. The phenomena governing gel layer formation and, consequently, solute release rate are water penetration, guar gum swelling, drug diffusion, and matrix erosion [19].

## CONCLUSION

The effects of solute solubility and release media have been addressed with regard to the swelling behavior and gel barrier formation of guar gum matrices. The dissolution rate of solutes from guar gum matrices was dependent on the presence of ions, pH and solute solubility under normal testing conditions. In most cases, the macromolecular relaxation led to a time dependent water uptake due to continuous polymer swelling. There was also a continuous decline in solute release rates due to a decreasing trend in the matrix tablet swelling rates.

The mechanisms of solute release from guar gum matrices were characterized by solute transport mainly due to the relaxation of the polymer. The rate of diffusion through the gel layer depended on solute dissolution and negligible matrix erosion affecting the solute concentration gradient in gel layer. Thus, solute release was linked to gel layer dynamics.

Release area and swelling volumes were dependent on the square root of time, indicating Fickian transport. This was attributed to the small size of water molecules relative to the pore space in the network. Further, caffeine transport was facilitated by the large solubility and concentration gradient, and polymer relaxation occurred leading to a Fickian diffusion. Conversely, salicylic acid having a very low solubility showed the lowest release in the media.

## REFERENCES

- [1] Qiu Y, Chen Y, Zhang G, Liu L, Porter W(eds). Rational design of oral modified-release drug delivery systems. In: Developing solid oral dosage

- forms Pharmaceutical theory and practice. *Elsevier*; 2009. p. 469–99.
- [2] Vasquez MJ, Pérez JB, GómeJL, Martinez R, Souto C, Concheiro A. Influence of technological variables on release of drugs from hydrophilic matrices. *Drug Dev Ind Pharm*. 1992;81:1355–75.
- [3] Huber HE, Dale LB, Christenson GL. Utilization of hydrophilic gums for the control of drug release from tablet formulations, I. Disintegration and dissolution behavior. *J Pharm Sci*. 1966;55:974–6.
- [4] Melia CD. Hydrophilic matrix sustained release systems based on polysaccharide carriers. *Crit Rev Ther Drug Carr Syst*. 1991;8:395–421.
- [5] Tripathy S, Das MS. Guar gum: Present status, and applications. *JPSI*. 2013;2(4):24–8.
- [6] Mudgil D, Barak S, Khatkar BS. Guar gum: Processing, properties and food applications-A review. *J Food Sci Technol*. 2014;51(3):409–18.
- [7] Ritger PL, Peppas NA. A simple equation for description of solute release. I. Fickian and non Fickian. *J Control Release*. 1987;5(1):23–6.
- [8] Ritger PL, Peppas NA. A simple equation for description of solute release. II. Fickian and anomalous release from swellable devices. *J Control Release*. 1987;5(1):37–42.
- [9] Peppas NA, Sahlin JJ. A simple equation for description of solute release. II. Fickian and anomalous release from swellable devices. *Int J Pharm*. 1989;57:169–72.
- [10] Ranga KV, Padmalatha K, Buri P. Cellulose matrices for zero order release of soluble drugs. *Drug Dev Ind Pharm*. 1988;14:2299–320.
- [11] Peppas NA. Analysis of Fickian and non Fickian drug release from polymer. *Pharm Acta Helv*. 1985;60:110–1.
- [12] Möckel JE, Lippold BC. Zero order drug release from hydrocolloid matrices. *Pharm Res*. 1993;10:1066–70.
- [13] Hancock B, Zografi G. The relationship between the glass transition temperature and the water content of amorphous pharmaceutical solids. *Pharm Res*. 1994;11:471–7.
- [14] Peppas NA, Colombo P. Analysis of drug release behavior from swellable polymer carriers using the dimensionality index. *J Control Release*. 1997;45:35–40.
- [15] Colombo P. Swelling controlled release in hydrogel matrices for oral route. *Adv Drug Del Rev*. 1993;11:37–57.
- [16] Colombo P, Bettini R, Massimo G, Catellani P, Santi P, Peppas NA. Drug diffusion front movement is important in drug release control from swellable matrix tablets. *J Pharm Sci*. 1995;84:991–7.
- [17] Doelker DE. Cellulose derivatives. *Adv Polym Sci*. 1994;107:199–265.



- [18] Ferrero C, Bruneau N, Barra J, Alfonso D, Doelker E. Hydrophilic cellulose derivates as drug delivery carriers: Influence of substitution type on the properties of compressed matrix tablets. In: Wise DL., editor. *Handbook of Pharmaceutical Controlled Release Technology*. Marcel Dekker Inc; 2000. p. 1–30.
- [19] Abrahamsson B, Alpsten M, Bake B, Larsson A, Sjögren J. In vitro and in vivo erosion of two different hydrophilic gel matrix tablets. *Eur J Pharm Biopharm*. 1998;46:69–75.

**Chapter 6**

## **THE APPLICATION OF PHYTOESTROGENS IN THE COSMETIC INDUSTRY**

***Alicja Kapuścińska, Anna Olejnik and Izabela Nowak\****

Adam Mickiewicz University in Poznań,  
Faculty of Chemistry, Poznań, Poland

### **ABSTRACT**

Phytoestrogens, known also as “youth hormones”, are interesting cosmetic innovative ingredients. These compounds are structural analogues of 17- $\beta$ -estradiol, a steroid and estrogen sex hormone. The most important and well-known class of phytoestrogens are isoflavones. Other classes of phytoestrogens such as lignans, stilbenes and coumestans have also been identified. It is expected that phytoestrogens exhibit anti-aging activity and may be applied in cosmetic products in glycoside forms of isoflavones. Much effort has been made to investigate the potential use of phytoestrogens as cosmetic active ingredients. Topical application of these compounds may be helpful in protecting the skin from free radicals and loss of firmness caused by decreasing collagenase activity. According to literature, the most significant isoflavone, genistein, has an inhibitory activity against protein called tyrosine kinase. Kinases are involved in transduction of signal activated by free radicals and inflammatory cytokines, that leads to the expression of enzymes responsible for the degradation of collagen and elastin, being a key factor causing a decrease

---

\* Corresponding Author Email: nowakiza@amu.edu.pl.

in elasticity of skin and skin aging, therefore, the aim of this chapter is to present the application of different phytoestrogens in cosmetic formulations. The information regarding the effectiveness of phytoestrogens as cosmetic active ingredient is included. The goal of this chapter is to review both *in vitro* and *in vivo* studies that were carried out to check the activity of phytoestrogens used in topical products.

## INTRODUCTION

Cosmetic chemistry and cosmetology are the two dynamically developing areas of research of great significance for cosmetic industry. According to recent developments, cosmetic producers tend to replace the synthetic components with natural substances of plant origin. A great cosmetic discovery of the 21<sup>st</sup> century are phytoestrogens which are substances of plant origin that are structural analogues of human estrogens known also as the “youth hormones” [1]. Estrogens are responsible for the young and healthy look of the skin as well as for the growth and density of hair. Because of a considerable drop in the production of these hormones in the menopause a hormone replacement therapy (HRT) is recommended in which estrogens are administered orally to balance the hormones levels in the organism [2]. Unfortunately, not all women can be treated with HRT. There is also a possibility of using supplements containing plant-origin analogues of estrogens although of weaker effect, phytoestrogens [3]. Positive effects of oral administration of phytoestrogens have stimulated studies of their influence on the skin. This paper presents a review of literature data on the use and effectiveness of phytoestrogens applied for the skin care.

## DEFINITION, DIVISION AND STRUCTURE OF PHYTOESTROGENS

Phytoestrogens are non-steroid organic compounds of plant origin. Their name alludes to their specific type of activity resembling that of the female sex hormone estrogens [1]. Also their chemical structures resemble those of estrogens because phytoestrogens have a pair of hydroxyl group and a phenolic ring [4]. They are recommended to women in the menopause period who cannot be administered synthetic estrogen preparations [3]. The terms “phytoestrogens” and “phytohormones” are often erroneously interchangeably used because of their incorrect definitions. In plants phytoestrogens are

synthesized from phenylpropanoids and simple phenols and show fungicidal, antioxidant activity, are stress signalers and protect plants against ultraviolet radiation. Phytohormones are the substances acting as hormones in plants but they have no effect on the endocrine system of human organism [5, 6].

On the basis of their chemical structure, phytoestrogens can be classified into different categories: flavonoids (flavones, flavonols, flavanones, and isoflavonoids), lignans, stilbenes, coumestans, chalcones and other miscellaneous classes [4, 7]. The most important and best known classes of phytoestrogens are isoflavones belonging to flavonoids. These compounds are considered to be the most interesting to cosmetic researchers. They are polyphenols whose main structural element is diarylpropane moiety [8]. The main representatives of isoflavones are genistein (4',5,7-trihydroxyisoflavone), daidzein (4',7-dihydroxyisoflavone) and glycitein (4',7-dihydroxy-6-methoxyisoflavone). These compounds have hydroxyl groups at positions 7' and 4' in the configuration analogous to that of 17- $\beta$ -estradiol molecule [9, 10] – Figure 1. Glycitein is a phytoestrogen showing weak estrogen activity relative to that of genistein and daidzein. This compound makes 5–10% of the total content of isoflavones in soy products. Estrogen-like activity of genistein isolated from dyer's greenweed (*Genistatinctoria*) was for the first time described in 1931. The richest source of phytoestrogens from the group of isoflavones is soybean (*Glycine max*). Roasted seeds of soy and soy flour contain from 130 to 170 mg isoflavones in 100 grams of raw products. The food products based on soy, such as soya sauce or soya pasta, are poorer in isoflavones. Soy is not the only source of phytoestrogens from this class, the others are the seeds of leguminous plants, cereals and some fruit and vegetables which can contain these substances in the amount up to 1 mg per 100 g of raw product [1, 11].

Food products contain also other compounds from the group of isoflavones such as formononetin, biochanin A, showing high ability to selective modulation of the estrogen receptor, the so-called selective estrogen receptor modulators (SERM) – Figure 1 [12]. Flavonols showing estrogen-like activity include kaempferol and quercetin, but their effect is weaker than that of isoflavones [5].

Lignans make another class of phytoestrogens whose main representatives are pinoresinol, lariciresinol, secoisolariciresinol and matairesinol. These compounds occur as dimers that in human organisms are present as glycosides [3]. Linseeds are the richest source of lignans, mainly secoisolariciresinol, containing on average 80 mg of active substance in 100 grams of raw product [13]. Lignans in the form obtained from plants do not show estrogen activity but

in human organism they are transformed to enterolactone and enterodiol which show estrogen-like activity [1].

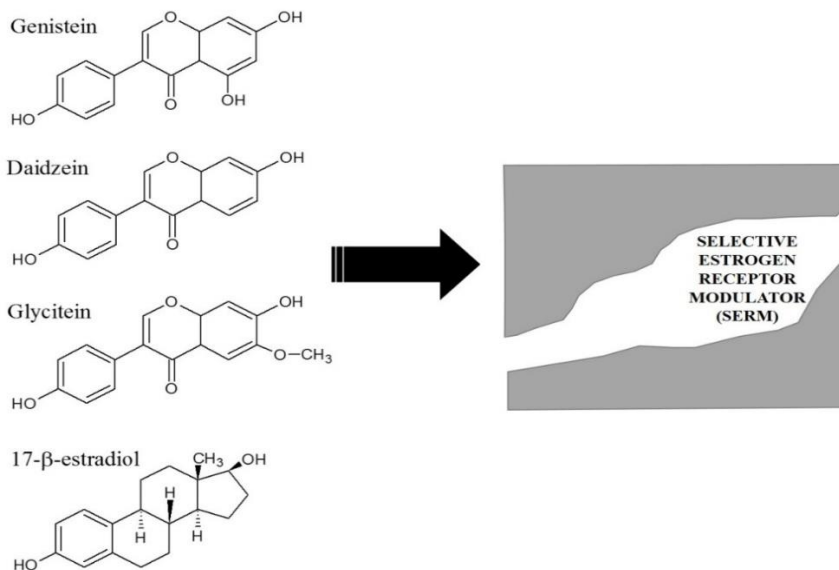


Figure 1. Genistein, daidzein, glycitein – chemical structures of structural analogues of 17-β-estradiol.

Stilbenes make another group of organic compounds of estrogen-like activity, they are produced by plants in response to stress or microbial infection [4]. The most important in this group is resveratrol (Figure 2), which is a polyphenol derivative of stilbene, present in two isomers, *cis* and *trans*. The spatial structure, similar to estradiol, enables resveratrol to bind to human estrogen receptors [14]. The richest source of this compound is red grapes, although it also occurs in peanuts and skin of mulberry and blackcurrant (up to 1 mg in 100 grams of raw product) [15]. In plants resveratrol is synthesized with the use of stilbene synthase enzyme [16] and it occurs in the form of *trans* isomer. It is an effective antioxidant, capable of activating SIRT1 gene (sirtuinprotenine 1) [17]. SIRT1 is the enzyme used for deacetylation of proteins that contribute to cellular regulation (reaction to stress, longevity) [18]. Under the effect of UV radiation *trans*-resveratrol (Figure 2) is transformed into *cis* isomer that does not show biological activity. Only a few compounds from the group of coumestans are classified as phytoestrogens, the strongest estrogen-like activity shows coumestrol. It is found in lucerne (*Medicago sativa*) shoots, soy sprouts and spinach (*Spinaciaoleracea*) leaves [1].

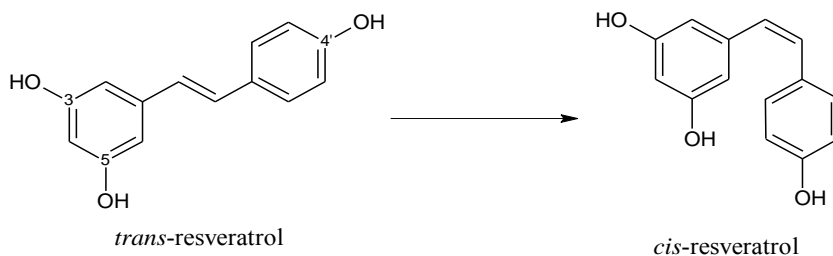


Figure 2. Chemical structure of *trans*- and *cis*-resveratrol.

## MECHANISM OF PHYTOESTROGENS ACTIVITY

The mechanism of phytoestrogens activity, mainly of the activity of isoflavones (genistein, daidzein, glycitein) is based on the structural similarity to 17- $\beta$ -estradiol (Figure 1). Phytoestrogens enter the cell through diffusion and inside the cell they attach to the estrogen receptor. The compounds are able to attach both to  $\alpha$  (ER- $\alpha$ ) and  $\beta$  (ER- $\beta$ ) forms of the estrogen receptor, although the genistein affinity to ER- $\beta$  is about 30 times greater than to ER- $\alpha$ . As a result of the interaction the receptor is activated and gives the so-called estrogen response [19]. To produce the effect comparable to that stimulated by the physiological concentration of 17- $\beta$ -estradiol the concentration of genistein should reach about 100 nM. Genistein shows the highest affinity to estrogen receptor from among all isoflavones. The affinity of the other substances from this group is by 100–500 times lower than that of 17- $\beta$ -estradiol [20]. Isoflavones can accumulate in the human organism. Their presence was detected in the tissue of mammary gland in women and in prostate in men [21].

## THE EFFECT OF ESTROGENS ON PHYSIOLOGY AND LOOK OF THE SKIN

The ageing of skin is a progressive physiological process that cannot be stopped but can be slowed down. The rate of this process depends on many factors including diet, lifestyle, habits, genes and skin care [18]. The endocrine system activity is an important and well-known factor affecting skin ageing. Of key importance are estrogens (sex hormones) including estradiol, estrone and estriol. The concentration of estrogens decreases in the menopause period [22] which is accompanied by increased intensity of ageing processes, both those

related to the age and to the environmental factors. It has been established that estrogen and its derivatives influence the correct skin pigmentation, vascularization, elasticity and ability to control the excessive through-epithelium water loss (TEWL) [22-24]. Moreover, these hormones regulate the growth of hair and healing of injuries [25]. Estrogens can affect the cell through the three main mechanisms, through interaction with estrogen receptors (ER) present in the cell membrane, through a direct influence on gene expression by nuclear estrogen receptors and through indirect influence on gene expression by nuclear estrogen receptors. Estrogen receptors (ER) occur in two forms as ER- $\alpha$  and ER- $\beta$ . The two receptors are proteins, ER- $\alpha$  is composed of 595 amino acids, while ER- $\beta$  of 485 amino acids. Both receptors are built of 6 similar action domains, including the ligand-bonding domain and the DNA bonding domain [20, 26-29]. The effect of estrogens on the skin is also related to the presence of ER- $\beta$  in the skin [29]. In the skin of the face the expression of estrogen receptors is much greater than in the skin of the breasts or thighs [21]. In the hairy skin of the head ER- $\beta$  were found to dominate, which was established by the immunohistochemical method using specific antibodies against ER- $\alpha$  and ER- $\beta$  [30]. A large number of ER- $\beta$  was found in the basal (*stratum basale*), cornified (*stratum corneum*) and granular (*stratum granulosum*) layers of the epidermis and in the papillary region of the dermis [31]. The studies based on the Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) revealed the presence of mRNA for the ER- $\alpha$  and ER- $\beta$  proteins in human fibroblasts (dermis cells responsible for secretion of elastin and collagen fibers and glycosaminoglycans) which evidences the interaction of estradiol on the skin via direct regulation of fibroblast activity [32]. The mechanism of estrogen induced production of collagen by fibroblasts has not been fully understood yet, although the effect of estrogens (and environmental factors) on the level of TGF- $\beta$  – a growth agent promoting production of collagen by fibroblasts, has been postulated [33]. According to literature data, estrogens deficiency causes weakening of TGF- $\beta$  signal transfer to the cell nucleus through a direct effect on the level of growth agent, which leads to reduced level of syntheses of collagen, fibronectin and proteoglycans [21]. It has been reported that exogenous estrogens influence the moisture content of the skin and its elasticity as a result of reduced transepidermal water loss (TEWL) and increased concentration of lipids in the hydro-lipid barrier of the skin. It is also claimed that estrogens inhibit apoptosis of keratinocytes as a consequence of activation of membrane receptors, which opens the non-classical signal transmission [21].

## EFFECTIVENESS OF PHYTOESTROGENS AS COSMETIC ACTIVE INGREDIENTS

The above-described biological activity of phytoestrogens has inspired the attempts at their use as cosmetic active ingredients. The majority of phytoestrogen-based cosmetic products for ageing skin care contain the glycoside forms of isoflavones [34]. A number of experiments have been performed to evaluate the effectiveness of phytoestrogens as cosmetic active ingredients. The examples of these studies are enlisted in Table 1. The externally applied phytoestrogens protect the skin against unfavorable oxidative processes and loss of firmness as a result to reducing the activity of collagenase that catalyzes decomposition of supporting fibers in dermis [35]. It was also proved that isoflavones could be applied in the dermocosmetics because they enhance the production of hyaluronic acid [36].

**Table 1. Examples of studies on effectiveness of phytoestrogens**

Sample type	Assessment	Methods	Results	Ref.
o/w emulsion containing soy extract (genistein and daidzein)	Effects on skin aging parameters after exposure to UV-A	<i>In vitro</i> Fibroblast culture	Increase in collagen and HA synthesis	37
Soy extract and soy isoflavones	Inhibition of retinoid-induced epidermal hyperplasia	<i>In vitro</i> Human skin organ culture	Decrease in hyperplasia and keratinocyte proliferation. Increase in synthesis of type I procollagen	38
Isoflavones solutions	Photoprotection after UV-B exposure	<i>In vivo</i> Pig skin	Decrease in erythema and sunburn cell formation	39
Gel containing concentrated soy extract (4 wt. %genistein)	Effects on skin parameters, anti-aging effect	Human studies	Increase in epidermal thickness and dermal capillary vessel, no gain in number of fibroblasts and dermal papillae	40
Cream containing 1% (w/v) <i>P. candollei</i> var. <i>mirifica</i> extract	Effects on skin parameters	Animal studies (rat)	Increase in skin hydration and elasticity	41



According to literature, genistein shows inhibiting activity towards tyrosine protein kinase. Kinases are engaged in transmission of the signals activated by free radicals and inflammatory cytokines, which leads to the expression of enzymes responsible for degradation of collagen and elastin, which is a fundamental factor responsible for a decrease in the skin elasticity and its ageing [42].

## ISOFLAVONES IN *IN VITRO* STUDIES

To check the effect of genistein on human fibroblasts the *in vitro* study was performed in which the cell colonies were treated with a genistein solutions of different concentrations and the progress in cell proliferation was evaluated by the MTT test. The test is based on measurement of effectiveness of energy transformations in mitochondria. The cell viability is measured by reduction of tetrazole salt (MTT which is 3-(4,5-dimethyl-tiazol-2-il)-2,5-difenyltetrazole bromide, a water soluble substrate of white or yellow color) to water insoluble formazane (dark blue in color). The amount of reduced MTT is proportional to the oxidative activity of the cell mitochondria, and in strictly controlled experimental conditions – to the number of metabolically active (alive) cells in the population [43]. The level of reactive oxygen species was determined by flow cytometry. Biochemical methods were applied to analyze the cell content of MDA (malonic dialdehyde) indicating the integrity of cell membranes. MDA is one of the final products of peroxidation of polyunsaturated fatty acids in cells, so an increased number of free radicals causes an increase in the content of MDA [44]. The activity of superoxide dismutase in cells was also assessed. The results evidenced a considerable increase in the cell proliferation in response to the treatment with a genistein solution of concentrations 0.0625-0.25 mg/L. The observed reduced level of reactive oxygen species confirmed the antioxidant activity of this compound. Moreover, genistein in the above specified concentration was found to inhibit expression of MDA. However, the expression of superoxide dismutase increased when fibroblasts were treated with genistein solution of 0.0625-0.25 mg/L concentration. The above results of *in vitro* studies confirmed that genistein has a positive effect on the functioning of human fibroblasts so it can be applied in anti-ageing cosmetic products [35].

Furthermore, Sudel et al. [37] observed that collagen synthesis increased after *in vitro* treatment of human fibroblast with purified genistein. Miyazaki et al. [36] studied the effects of genistein and daidzein on the hyaluronic acid production in a transformed human keratinocyte culture. Additionally, the

experiments were also performed on hairless mouse skin after application of isoflavones for 2 weeks. In both studies the enhanced production of hyaluronic acid was observed. Soy isoflavones can be also potential candidates for protective agents against photodamage. The studies conducted by Huang et al. [45] proved that genistein and daidzein could act as free radical scavengers when keratinocytes were photodamaged. On the other hand, glycitein did not exhibit the protective activity against photodamage.

## ISOFLAVONES IN *IN VIVO* STUDIES

To establish the degree to which genistein and daidzein are absorbed through skin the *in vivo* study was carried out, with olive oil as the cosmetic medium. The concentrations of the isoflavones and their metabolites in the blood plasma were monitored by gas chromatography-mass spectrometry (GC-MS) method. The concentration of genistein in blood plasma was about 3 times higher than that of daidzein. After multiple applications at certain time intervals the concentrations of these metabolites decreased because of accumulation of phytoestrogens in the skin [46]. Another less invasive method allowing evaluation of the isoflavones ability to penetrate skin is the so-called tape-stripping. It is a simple method for evaluation of quality and efficiency of cosmetic and dermatological preparations. After topical application and a certain time left to skin penetration an adhesive tape is stuck and then removed from the skin. It contains cells from *stratum corneum* and some amount of the preparation studied. Then with the help of classical methods of chemical analysis it is possible to determine the percent of absorption of a given substance [47]. This method was applied to check the skin penetration ability of the isoflavones. Three types of formulations were prepared, in the first genistein aglycone was contained in liposomes, in the second one genistein aglycone was free and in the third genistein glycoside contained in liposomes. Each formulation was applied on the forearms of volunteers (50  $\mu$ l of formulation on 1 cm<sup>2</sup> of the skin). After 4 hours from the application the adhesive tape was stuck to the skin and then removed. The amount of the formula left on the tapes was removed by the solvent and analyzed by high-performance liquid chromatography (HPLC). The results proved that the active substance contained in liposomes penetrated the skin more easily than free substance. Moreover, minimum enzymatic activity of the *stratum corneum* of epidermis was observed as the glycoside form of genistein was not hydrolyzed to the biologically active form of aglycone. Unfortunately, genistein aglycone (the molecular form

without sugar residues) in the free form also showed poor ability to penetration of epidermis, which was related to its poor solubility in water as well as in lipid substances. Taking into account the above, the best formula to be applied in cosmetic products seems to be isoflavone aglycones contained in liposomes [48]. The safety of genistein use in cosmetic products was confirmed by patch tests. No allergenic or photoallergenic effect of the isoflavones studied was noted. The effect of genistein on the skin thickness was also studied. The cosmetic preparations with genistein containing 90 mg of the active substance in 1 kg of the product were applied on the skin of the forearm inside of 20 female volunteers of 55-64 years of age. For comparison the placebo was applied. In 2 or 3 months after the first application the skin thickness (ultrasonic probe measurement), elasticity (cutometer) and moisture content (corneometer) were measured. The skin treated with the genistein preparation was observed to grow in thickness by 11%, while the placebo caused scanty changes [46]. The application of genistein also resulted in an increase in elasticity and moisture content [48]. It has been shown in many studies, e.g., by measurements of concentration of collagen markers in the fluid from blisters made on the skin, skin thickness measurements by USG method and immunohistochemical measurements in skin collected in biopsy that estrogen deficiency related with menopause results in a reduction in the number of collagen fibers in the skin. These adverse changes were reversible after application of different forms of hormonal supplementation, including the through skin one [31, 49]. There are scarce reports that negate the beneficial effects of hormone replacement therapy on the skin thickness, synthesis of collagen or elastin [50].

## **APPLICATION OF PHYTOESTROGENS IN COSMETIC FORMULATIONS**

There are a lot of patents on skin care products containing isoflavones [51]. The companies that invest in products comprising isoflavones include the German firm Beiersdorf, the French one Pharmascience, Korean one Amorepacific and Kolmar [51]. Beiersdorf invented formulations comprising isoflavones intended for sebum reduction, antiperspirant and anti-ageing actions and limitation of sensitive skin irritation. On the other hand, the French company Silab obtained the isoflavone aglycones from iris rhizomes of plants belonging to the genus *Florentine* and afterwards introduced them into creams and gels. Formulations containing isoflavones are available on the market as

anti-ageing cosmetic products [51] that are supposed to increase skin elasticity and minimize wrinkles [52]. The examples of products containing phytoestrogens are presented in Table 2.

**Table 2. Examples of products containing phytoestrogens available on the market**

<b>Product name</b>	<b>Producer</b>
Nivea Visage Vital multi effect anti-age – cream	Beiersdorf
Avenno® cream	Johnson and Johnson
Emerita, phytoestrogen® body cream	Emerita company
Rojukissprotox day cream SPF 50 PA+++	Aisance Co., Ltd
Flavonex® – cream	Auriga International
Isoflavonia®– body lotion	Janssen Cosmetics
Soy Relief® Intensive Cream and Soy Relief® Mild Lotion	Amorepacific
Neovadiol®	Vichy

One of the skin care product including isoflavonoids, Neovadiol® was tested on 234 women in the maximum age of 65 years and at least 3 years since menopause [59, 60]. The results of these studies proved that skin dryness and roughness greatly improved at treated areas compared to untreated areas. Additionally, facial wrinkles and skin looseness were reduced. The European Vichy®, American Aveeno® and SkinCeuticals® and Brazilian Adcos®, Natura®, and Payoit® are companies, which invest in the production of cosmetics containing soy extracts [61]. However, in some cases the extracts are introduced to cosmetics in conjugated forms that reduce their biological action due to lower skin penetration ability. Therefore, as alternative to conventional systems, the isoflavone aglycones were incorporated into liposomes, micro and nanosystems and were also complexed with cyclodextrins [53, 54, 62, 63]. New technological strategies to increase the release of isoflavones are presented in Table 3.

It should be mentioned that the active isoflavones for skin care products must be in the form of aglycones. These isoflavones forms have great capacity to be absorbed in skin and have potential therapeutic effects when compared to conjugated forms [64]. Schmid et al. [65] have proposed the liposomal aglycone preparation that exhibited high bioavailability in the skin. These liposomal systems can be applied in various topical formulations such as creams, lotions and gels.

**Table 3. Different technologies for topical systems containing isoflavones**

Type of isoflavone	Technological system	Other components	Ref.
Genistein	Liposome	Phospholipids, polysorbate 80	53
	Nanoemulsion	Medium chain triglycerides, egg lecithin	54
	Nanocapsule	Poly (acid lactic)	55
Isoflavone aglycones	Solid lipid nanoparticle	Softsan 601, polysorbate 20	56
Daidzein	Dendrimers	Poly(amidoamine) or Poly(propylene imine)	57
	Cyclodextrin (CD)	$\beta$ -CD, methyl- $\beta$ -CD, hydroxypropyl- $\beta$ -CD	58

On the other hand, isoflavones are also promising compounds for anti-cellulite products, because they are supposed to enhance the skin tone and viscoelasticity. Isoflavones were combined with the rich in minerals algae extract to obtain efficient anti-cellulite preparation [65].

## PHYSICOCHEMICAL ANALYSIS OF PLANT EXTRACTS CONTAINING PHYTOESTROGENS

The plant extracts are more often used in cosmetic industry than pure phytoestrogen compounds mainly because of lower costs of production. Therefore, it is important to know exactly the composition of the extracts applied in cosmetic products. Progress in the methods of physicochemical analysis permitted obtaining isoflavones from plant material by extraction with the use of different solvents or mixtures. Some phytoestrogens (daidzein, genistein, coumestrol) can be obtained from soy seeds by extraction with a mixture of acetonitrile and water. Very effective proved to be the supercritical phase extraction of the soy seed flour [66]. In 1974, the use of gas-liquid chromatography (GLC) was proposed for identification of genistein and daidzein after their transformation into trimethylsilyl derivatives. In 1978, the analysis of genistein and 4',6,7-trihydroxyisoflavone by high-performance liquid chromatography (HPLC) was proposed. The extract from soy seeds (extraction by water and acetonitrile) is filtered off by glass fiber filter and the filtrate that contains phytoestrogens is analyzed by HPLC (C18 column)

coupled with a UV-Vis detector. The method is effective for the isoflavone concentrations not lower than 2 ppm [67]. Analysis of coumestrol is based on fluorescence detection, but the concentration of this compound should not be lower than 0.5 ppm [68]. Derivatives of phytoestrogens (genistein, daidzein and coumestrol) can be also extracted from soy roots by using dimethyl sulfoxide (DMSO) and then separated and isolated by HPLC. The eluate is then concentrated and individual compounds can be analyzed using a UV spectrophotometer (the spectral range of the substances analyzed in methanol was 200 - 450 nm, isoflavones show maximum absorption at 260 nm) or by gas chromatography coupled with mass spectrometry [69, 70]. Earlier methods permitted analyses of isoflavones mainly in the form of aglycones, which required hydrolysis of the compounds occurring in the form of glycosides. At present analysis of both aglycones and glycosides is possible. Acetylic and malonic glucosides are hydrolyzed to 7-O-glucosides with the use of 80% ethanol solution containing 1 mol of chloric acid to remove acetyl and malonic groups. Then, aglycones and 7-O-glucosides are analyzed by HPLC coupled with coulometric detection method [71].

## CONCLUSIONS

Phytoestrogens are plant-origin compounds that have been recently a subject of interest of cosmetic and pharmacological industry. Because of the diversity of biological activity, the compounds can show synergistic effect with some hitherto used therapeutic substances or can substitute them. On the basis of the results presented it can be concluded that phytoestrogens applied on the skin show antiaging activity. These compounds and genistein in particular stimulate the synthesis of fibroblasts and protect the skin against the loss of firmness caused by inhibition of collagenase activity which catalyzes decomposition of supporting fibers of dermis. Phytoestrogens also reduce the level of reactive oxygen species which proves their antioxidant activity. Toxicological analysis has proved that it is impossible to reach a toxically high level of these substances in the organisms by their application on the skin in the form of cosmetic products. Their use also does not bear a risk of side effects as the activity of these compounds is much weaker than that of natural estrogens.

## REFERENCES

- [1] Prescha, A. and Biernat, J. (2009). Wpływ fitoestrogenów pokarmowych na organizm człowieka (The influence of dietary phytoestrogens on the human body). *Bromat Chem Toksykol.*, 41, 941-948.
- [2] Wojnowska, D., Juskiewicz-Borowiec, M. and Chodorowska, G. (2006). Wpływ menopauzy na starzenie się skóry (The impact of menopause on skin aging). *Post Dermatol Alergol*, 3, 149-56.
- [3] Bińkowska, M., Pietrzak, B. and Dębski, R. (2005). Hormonalna terapia zastępcza w grupie kobiet polskich w wieku 45–54 lat. Wiedza, opinia, stosowanie (Hormone replacement therapy in a group of Polish women aged 45-54 years. The knowledge, expertise, use). *Przegl Menopauz*, 2, 19-27.
- [4] Yingngam, B.&Rungseevijitprapa W. (2012). Molecular and clinical role of phytoestrogens as anti-skin-ageing agents: A critical overview. *Phytopharmacology*, 3(2), 227-244.
- [5] Kraszewska, O., Nynca, A., Kamińska, B. and Ciereszko, R. (2007). Fitoestrogeny. I. Występowanie, metabolizm i znaczenie biologiczne u samicy (Phytoestrogens. I. Occurrence, metabolism and biological significance in females). *Post Biol Kom*, 34, 189-205.
- [6] Markiewicz, L., Garey, J., Adlercreutz, H. and Gurrpide, E. (1993). *In vitro* bioassays of non-steroidal phytoestrogens. *J Steroid BiochemMolBiol*, 45(5), 399-405.
- [7] Sirotkin, A.V. and Harrath, A.H. (2014). Phytoestrogens and their effect. *Eur J Pharmacol*, 741, 230-236.
- [8] Malinska, D. and Kiersztan, A. (2004). Flawonoidy – charakterystyka i znaczenie w terapii (Flavonoids-characteristics and importance in the therapy). *Post Bioch*, 50(2), 182-196.
- [9] Singh, P., Kumar, R., Sabapathy, S. N. and Bawa, A.S. (2008). Functional and edible uses of soy protein products. *Compr Rev Food Sci Food Saf*, 7, 14-28.
- [10] Cassidy, A., Bingham, S. and Setchell, K. (1994). Biological effects of a diet of soy protein rich isoflavones on the menstrual cycle of premenopausal women. *Am J Clin Nutr*, 60, 333-340.
- [11] Bijak, M., Połać, I., Borowiecka, M., Nowak, P., Stetkiewicz, T. and Pertyński, T. (2010). Izoflawony jako alternatywa dla terapii hormonalnej wieku menopauzalnego (Isoflavones as an alternative to hormone therapy of menopausal age). *Prz Menopauz*, 6, 402-406.

- 
- [12] Setchell, K. D. (2001). Soy isoflavones-benefits and risks from nature's selective estrogen receptor modulators (SERMs). *J Am Coll Nutr*, 20(5), 354-362.
- [13] Mazur, W. (1998). Phytoestrogen content in foods. *Bailliere's Clin. Endocrinol. Metab*, 12, 729-743.
- [14] Kocjan, R., Strzemski M., Sowa, I., Polski, A., Szwerc W., Świeboda, R. & Blicharski, T. (2011). Phytoestrogens – classification, occurrence and significance in the prevention and treatment of osteoporosis. *Annales Universitatis Mariae Curie – Skłodowska Lublin – Polonia*, XXIV (1) 23, 196-203.
- [15] Jang, M., Cai, L., Udeani, G. O., Slowing, K. V., Thomas, C. F., Beecher, C. W. and Pezzuto, J. M. (1997). Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science*, 275(5297), 218-220.
- [16] Schröder, J. (1999). The chalcone/stilbene synthase-type family of condensing enzymes. In D.H.R. Barton, K. Nakanishi (Eds.), *Comprehensive natural products chemistry*, (1) 749-771.
- [17] Knutson, M. D. and Leeuwenburgh, C. (2008). Resveratrol and novel potent activators of SIRT1: effects on aging and age-related diseases. *Nutr Rev*, 66(10), 591-596.
- [18] Motta, M. C., Divecha, N., Lemieux, M., Kamel, C., Chen, D., Gu, W. and Guarente, L. (2004). Mammalian SIRT1 represses forkhead transcription factors. *Cell*, 116(4), 551-563.
- [19] Barnes, S., Kim, H., Darley-USmar, V., Patel, R., Xu, J., Boersma, B. and Luo, M. (2000). Beyond ER $\alpha$  and ER $\beta$ : estrogen receptor binding is only part of the isoflavone story. *J Nutr*, 130(3), 656S-657S.
- [20] Kuiper, G. G., Enmark, E., Peltö-Huikko, M., Nilsson, S. and Gustafsson, J. A. (1996). Cloning of a novel receptor expressed in rat prostate and ovary. *Proceedings of the National Academy of Sciences*, 93(12), 5925-5930.
- [21] Wojas-Pelc, A., Nastalek, M. and Sułowicz, J. (2008). Estrogeny a skóra – spowolnienie procesu starzenia (Estrogen and the skin-slowng down the aging process). *Prz Menopauz*, 12, 314-8.
- [22] Piérard, G.E., Letawe, C., Dowlati, A. and Piérard-Franchimont, C. (1995). Effect of hormone replacement therapy for menopause on the mechanical properties of skin. *J Am Geriatr Soc*, 43, 662-665.
- [23] Beas, F., Vargas L., Spada, R.P. and Merchak, N. (1969). Pseudoprecocious puberty in infants caused by dermal ointment containing estrogens. *J Pediatr*, 75, 127-130.



- [24] Haenggi, W., Linder, H.R., Birkhaeuser, M.H. and Schneider, H. (1995). Microscopic findings of the nail-fold capillaries-dependence on menopausal status and hormone replacement therapy. *Maturitis*, 222, 37-46.
- [25] Ashcroft, G.S. and Ashworth, J.J. (2003). Potential role of estrogens in wound healing. *Am J Clin Dermatol*, 4, 737-43.
- [26] Fuqua, S. A., Schiff, R., Parra, I., Friedrichs, W. E., Su, J. L., McKee, D. D. and Moore, J. T. (1999). Expression of wild-type estrogen receptor  $\beta$  and variant isoforms in human breast cancer. *Cancer Research*, 59(21), 5425-5428.
- [27] Gustafsson, J. A. (2000). New insights in oestrogen receptor (ER) research—the ER $\beta$ . *Eur J Cancer*, 36, 16.
- [28] Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engström, O. & Carlquist, M. (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature*, 389 (6652), 753-758.
- [29] Kuiper, G. G., Lemmen, J. G., Carlsson, B. O., Corton, J. C., Safe, S. H., Van Der Saag, P. T. and Gustafsson, J. A. (1998). Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor  $\beta$ . *Endocrinology*, 139(10), 4252-4263.
- [30] Thornton, M. J., Taylor, A. H., Mulligan, K., Al-Azzawi, F., Lyon, C. C., O'Driscoll, J. and Messenger, A. G. (2003). Oestrogen receptor beta is the predominant oestrogen receptor in human scalp skin. *Exp Dermatol*, 12(2), 181-190.
- [31] Thornton, M. J. (2002). The biological actions of estrogens on skin. *Exp Dermatol*, 11(6), 487-502.
- [32] Haczynski, J., Tarkowski, R., Jarzabek, K., Slomczynska, M., Wolczynski, S., Magoffin, D. A. & Jakimiuk, A. J. (2002). Human cultured skin fibroblasts express estrogen receptor  $\alpha$  and  $\beta$ . *Int J Mol Med*, 10(2), 149-153.
- [33] Dobrzycka, B., Kinalski, M., Piechocka, D. & Terlikowski, S. J. (2009). The role of estrogens in angiogenesis in the female reproductive system. *Endokrynologia Polska*, 60(3), 210-214.
- [34] Msika, P. and Piccardi, N. (2005). Use of isoflavones for preparing topical compositions for promoting slimming, and related cosmetic treatment method US Patent 20050256061 A1.
- [35] Setchell, K. D. (1998). Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones. *Am J Clin Nutr*, 68(6), 1333-1346.

- 
- [36] Miyazaki, K., Hanamizu, T., Iizuka, R. and Chiba, K. (2002). Genistein and daidzein stimulate hyaluronic acid production in transformed human keratinocyte culture and hairless mouse skin. *Skin Pharmacol Appl Skin Physiol*, 15, 175-183.
- [37] Sudel, K.M., Venzke, K., Mielke, H., Breitenbach, U., Mundt, C., Jaspers, S. and Gallinat, S. (2005). Novel aspects of intrinsic and extrinsic aging of human skin: beneficial effects of soy extract. *Photochem Photobiol*, 81(3), 581-587.
- [38] Varani, J., Kelley, E. A., Perone, P. and Lateef, H. (2004). Retinoid-induced epidermal hyperplasia in human skin organ culture: inhibition with soy extract and soy isoflavones. *Exp Mol Pathol*, 77, 175-183.
- [39] Lin, J.Y., Tournas, J.A., Burch, J.A., Monteiro-Riviere, N.A. and Zielinski, J. (2008) Topical isoflavones provide effective photoprotection to skin. *Photodermatol Photoimmunol Photomed*, 24, 61-66.
- [40] Moraes, A.B., Haidar, M.A., Soares, J. J.M., Simoes, M.J., Baracat, E.C. and Patriarca, M.T. (2009). The effects of topical isoflavones on postmenopausal skin: double-blind and randomized clinical trial of efficacy. *Eur J Obstet Gyn Reprod Biol*, 146, 188-192.
- [41] Yingnam, B. (2011). Development of anti-skin-aging products containing phytoestrogenic extract using nanotechnology. UbonRatchathani University, UbonRatchathani, Thailand, pp. 1 -365.
- [42] Ullrich, A. and Schlessinger, J. (1990). Signal transduction by receptors with tyrosine kinase activity. *Cell*, 61(2), 203-212.
- [43] Gerlier, D. and Thomasset, N. (1986). Use of MTT colorimetric assay to measure cell activation. *J Immunol Methods*, 94(1), 57-63.
- [44] Gutteridge, J. and Quinlan, G. J. (1982). Malondialdehyde formation from lipid peroxides in the thiobarbituric acid test: the role of lipid radicals, iron salts, and metal chelators. *J Appl Biochem*, 5(4-5), 293-299.
- [45] Huang, Z. R., Hung, C. F., Lin, Y. K. and Fang, J. Y. (2008). *In vitro* and *in vivo* evaluation of topical delivery and potential dermal use of soy isoflavones genistein and daidzein. *Int J Pharm*, 364, 36-44.
- [46] Maheux, R., Naud, F., Rioux, M., Grenier, R., Lemay, A., Guy, J. and Langevin, M. (1994). A randomized, double-blind, placebo-controlled study on the effect of conjugated estrogens on skin thickness. *Am J Obstet Gynecol*, 170(2), 642-649.
- [47] Lademann, J., Jacobi, U., Surber, C., Weigmann, H. J. and Fluhr, J. W. (2009). The tape stripping procedure—evaluation of some critical parameters. *European Eur J Pharm Biopharm*, 72(2), 317-323.

- [48] Schmid, D. & Züllli, F. (2002). Topically applied soy isoflavones increase skin thickness. *Cosmetics and toiletries*, 117(6), 45-50.
- [49] Verdier-Sévrain, S. (2007). Effect of estrogens on skin aging and the potential role of selective estrogen receptor modulators. *Climacteric*, 10(4), 289-297.
- [50] Oikarinen, A. (2000). Systemic estrogens have no conclusive beneficial effect on human skin connective tissue. *Acta Obstet Gynecol Scand*, 79(4), 250-254.
- [51] Nemitz, M. C., Argenta, D. F., Koester, L. S., Bassani, L. V., Poser, G. L. and Teixeira, H.F. (2016). The international scenario of patents concerning isoflavones. *Trends Food Sci Tech*, 49, 85-95.
- [52] Kapuscinska, A. and Nowak, I. (2015). The use of phytoestrogens in anti-ageing cosmetics. *Chemik*, 69, 154-159.
- [53] Schmid, D., Züllli, F., Nissen, H.P. and Prieur, H. (2003). Penetration and metabolism of isoflavones in human skin. *Cosmet Toilet*, 188, 71-74.
- [54] Silva, A.P.C., Nunes, B.R., De Oliveira, M.C., Koester L.S., Moayorga, P., Bassani, V.L. and Teixeira, H.F. (2009). Development of topical nanoemulsions containing the isoflavone genistein. *Pharmazie*, 64, 32-35.
- [55] Zampieri, A.L.T.C., Ferreira, F.S., Resende, E.C., Gaet, M.P.N., Diniz, D.G.A., Taveira, S.F. and Lima, E.M. (2013). Biodegradable polymetricnanocapsules based on poly(DL-lactide) for genistein topical delivery: obtention, characterization and skin permeation studies. *J Biomed Nanotechnol*, 9, 527-534.
- [56] Deshmukh, K. and Amin, P. (2013). Formulation and evaluation of solid-lipid nanoparticle based 0.1% Soy isoflavone dermal gels. *J Pharm Bio Sci*, 1, 7-18.
- [57] Zhao, C., Wang, Y., Su, Y., Zhang, H., Ding, L., Yan, X., Zhao, D., Shao, N., Ye, X. and Cheng, Y. (2011). Inclusion complexes of isoflavones with two commercially available dendrimers: solubility, stability, structures, delivery behaviors, cytotoxicity and antioxidant activities. *Int J Pharm*, 421, 301-309
- [58] Borghetti, G.S., Pinto, A.P., Lula, I.S., Sinisterra, R.D., Teixeira, H.F. and Bassani, V.L. (2011). Daidzein/cyclodextrin/hydrophilic polymer ternary systems. *Drug Dev Ind Pharm*, 37, 886-893.
- [59] Santor, P.G. (2006). Skin treatments and dermatological procedures to promote youthful skin. *Clin Interv Aging*, 1(1), 51-56
- [60] Bayerl, C. and Keil, D. (2002). Isoflavonide in der Behandlung der Hautalteringpostmenopausaler Frauen (Isoflavonoids in the treatment of skin aging in postmenopausal women). *Akt Dermatol* 28 (S1), 14-18.

- 
- [61] Nemitz, C. M., Moraes, R.C., Koester, L.S., Bassani, V. L., Poser, G.L. and Teixeira, H.F. (2015). Bioactive soy isoflavones: extraction and purification procedures, potential dermal use and nanotechnology-based delivery systems. *Phytochem Rev*, 14, 849-869.
- [62] Kitagawa, S., Inoue, K., Teraoka, R. and Morita, S.Y. (2010). Enhanced skin delivery of genistein and other two isoflavones by microemulsion and prevention against UV irradiation-induced erythema formation. *Chem Pharm Bull*, 58, 398-401.
- [63] Xavier, C.R., Silva, A. P.C., Schwingel, L.C., Borghetti, G.S., Koester, L.S., Mayorga, P., Teixeira, H.F. and Bassani, V.L. (2010). Improvement of genistein content in solid genistein/ $\beta$ -cyclodextrin complexes. *Quim Nova*, 33, 587-590.
- [64] Izumi, T., Piskula, M.K., Osawa, S., Obata, A., Tobe, K., Saito, M., Kataoka, S., Kubota, Y. and Kikuchi, M. (2000). Soy isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans. *J Nutr*, 130, 1695-1699.
- [65] Schmid, D., Reto, M. and Züllig, F. (2001). Dermatological application of soy isoflavones to prevent skin ageing in postmenopausal women, *Cosmetics and Toiletries*, 1, 146-151.
- [66] Rostagno, M. A., Araújo, J. and Sandi, D. (2002). Supercritical fluid extraction of isoflavones from soybean flour. *Food Chem*, 78(1), 111-117.
- [67] Wang, G., Kuan, S. S., Francis, O. J., Ware, G. M. and Carman, A. S. (1990). A simplified HPLC method for the determination of phytoestrogens in soybean and its processed products. *J Agric Food Chem*, 38(1), 185-190.
- [68] Naim, M., Gestetner, B., Zilkah, S., Birk, Y. and Bondi, A. (1974). Soybean isoflavones, characterization, determination and antifungal activity. *J Agric Food Chem*, 22, 806-810.
- [69] Barnes, S., Coward, L., Kirk, M. and Sfakianos, J. (1998). HPLC-mass spectrometry analysis of isoflavones. *Exp Biol Med*, 217(3), 254-262.
- [70] Porter, P. M., Banwart, W. L. and Hassett, J. J. (1985). HPLC isolation and GC-MS identification of genistein, daidzein, and coumestrol from unhydrolyzed soybean root extracts. *Environ Exper Bot*, 25(3), 229-232.
- [71] Peñalvo, J. L., Nurmi, T. & Adlercreutz, H. (2004). A simplified HPLC method for total isoflavones in soy products. *Food Chem*, 87(2), 297-305.



*Chapter 7*

## **THE POTENTIAL ROLE OF PHYTOESTROGENS IN HORMONAL REPLACEMENT THERAPY**

*Alicja Kapuścińska, Anna Olejnik and Izabela Nowak\**

Adam Mickiewicz University in Poznań,  
Faculty of Chemistry, Poznań, Poland

### **ABSTRACT**

Menopause is the time in most women's lives when menstrual periods stop permanently. This process typically occurs between 45 and 55 years of age and entails accelerating aging of body. It is caused by a decrease in estrogens production by the ovaries. In order to alleviate the signs of menopause, oral supplementation of estrogens may be recommended. However, according to recent results, not all women can be treated with medicines containing estrogens. For those who cannot, an oral supplementation with phytoestrogens may be considered. The most important and well-known class of phytoestrogens are isoflavones. Phytoestrogens are natural plant hormones that are structural analogues to 17- $\beta$ -estradiol. The mechanism of action of phytoestrogens, especially isoflavones, is based on structural similarity to 17- $\beta$ -estradiol. In this article we discuss the potential application of phytoestrogens as an alternative to

---

\* Corresponding Author Email: [nowakiza@amu.edu.pl](mailto:nowakiza@amu.edu.pl).

hormone replacement therapy. The evidence of isoflavones effectiveness in the treatment for menopausal symptoms is reviewed.

## **INTRODUCTION**

Menopause is the natural biological process in a woman's life when the function of the ovaries ceases. There are many symptoms of menopause, some of which may be alleviated by hormonal replacement therapy (HRT). This therapy is based on taking products containing sex hormones to supplement low concentration of sex hormones in women's body and hence eliminate many symptoms and effects of menopause [1]. However, not every woman's health is good enough to take hormones and additionally, HRT therapy may increase the risk of different diseases such as ovarian and breast cancers [2, 3]. Therefore it is important to replace exogenous hormones with their natural, plant counterparts. For example, phytoestrogens may be used for this purpose. These compounds are of plant origin and show activity similar to that of 17- $\beta$ -estradiol, female sex hormone [4]. In this article a literature review on menopause symptoms, the role and contraindications of HRT use is presented. Moreover, the potential application of phytoestrogens to alleviate the symptoms of climacteric is also presented.

## **MENOPAUSE – DEFINITION**

Menopause, also known under the term climacteric, is the natural biological process in every women's life. Menopause is connected with the ageing process of women's body and typically occurs between 45 and 55 years of age. It must be mentioned that perimenopausal, menopausal and postmenopausal periods may be distinguished. Perimenopause is the period when the biological, endocrine, and clinical features of climacteric begin. During this period a woman's body makes its natural transition toward menopause. Perimenopause is also called the menopausal transition. According to the North American Menopause Society, this transition can last for four to eight years [5]. During the menopausal transition and after menopause, women can experience a wide range of psychological, systemic, transitional symptoms [6, 7]. After this period, menopause occurs. During this process the menstrual period stops. It means that after climacteric women are no longer able to have children [8]. Menopause begins, when the menstrual cycles are still regular but the interval between

cycles gets longer and ovulation may not occur in each cycle. Moreover, the level of some hormones fluctuates [9]. After climacteric, postmenopause occurs. These are the years after menopause, during which menopausal symptoms frequently resolve for most women. However, the decrease in estrogen level may cause the risk for example osteoporosis and heart disease in postmenopausal women [10].

## SYMPTOMS OF MENOPAUSE

Every woman may feel the changes associated with menopause in different intensity, however the symptoms of this period are typical and include hormonal, physical and physiological aspects (Figure 1).

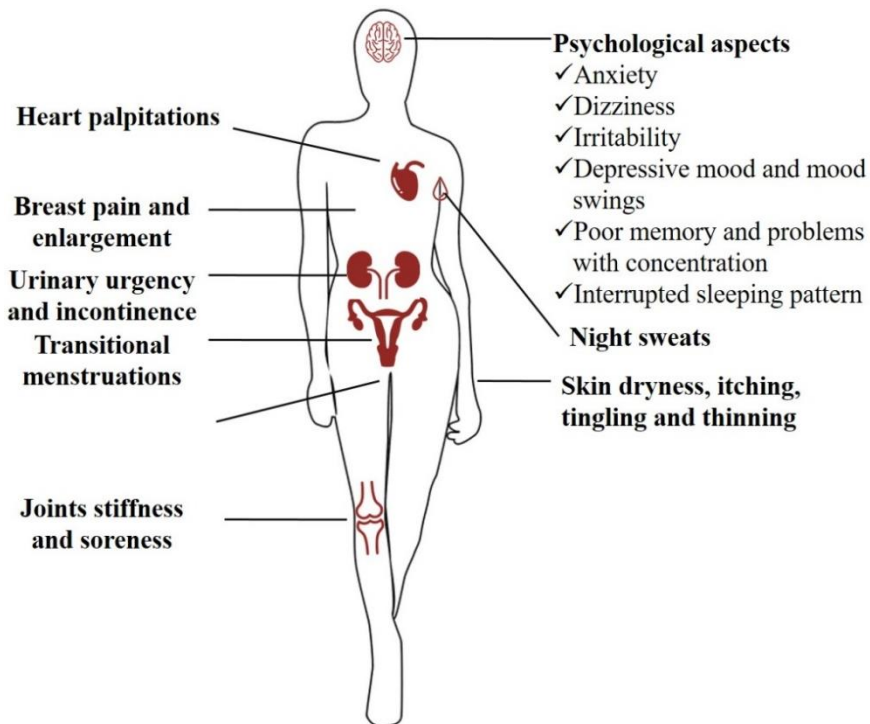


Figure 1. Symptoms of menopause.



## **Hormonal Changes in the Climacteric Transition**

In healthy women, the menopause transition is mainly caused by the loss of ovarian follicular activity. Climacteric includes changes in central neuroendocrine and in ovary physiology, such as profound decline in follicle numbers [11]. Follicular activity of ovaries may be determined by the level of follicle-stimulating hormone (FSH), which is a glycoprotein polypeptide-hormone, synthesized and secreted by the gonadotropic cells of the anterior pituitary gland. FSH regulates the development, growth, pubertal maturation, and reproductive processes of the body. This hormone stimulates maturation of follicles in ovaries as well as estrogen secretion and in the granulosa cells of follicles [12]. The increase in FSH level may be caused by declining levels of inhibin B, a direct marker of ovarian aging, which is a dimeric protein that reflects the reduction of ovarian follicle numbers. In women without the uterus, menopause or postmenopause can be identified by a blood test showing a very high FSH level [13]. The level of estrogen (a steroid sex hormone and the primary female sex hormone), remains relatively unchanged or tends to rise with age until the onset of the menopausal transition [14]. Moreover, the production of progesterone (sex hormone that maintains early pregnancy) stops during menstrual cycles when there is no ovulation and after final menstrual period [15].

## **Physical Changes**

As already mentioned, during perimenopause, menstrual patterns tend to be reduced to 2-7 days, however longer cycles still remain possible [16]. Menstrual bleeding may become irregular and more or less intensive. During perimenopausal period dysfunctional uterine bleeding may also occur [17]. In the women in postmenopause, genital bleeding may be related to polyp or lesion as well as vaginal atrophy or functional endometrial response. However, bleeding in postmenopause time may be a symptom of malignant diseases, so it may require appropriate examination [18, 19]. Besides the changes in the endometrium, which is usually the main source of bleeding, other symptoms of menopause such as vaginal dryness or atrophic vaginitis may occur. Atrophic vaginitis means thinning of the vagina, cervix and vulva as well as outer urinary tract, including shrinking and loss in elasticity of both the outer and inner genital areas [20]. Other physical symptoms of menopausal period appear in the skin (dryness, itching, tingling skin), the breast (pain and enlargement), and include

headaches and back pain, heart palpitations, hot flashes, heavy night sweats and interrupted sleeping cycles and finally, lack of energy, dizziness and urinary problems [16, 21-23].

## **Psychological Changes**

Menopause is not only associated with changes in body functioning, but it has also influence on women's psychology. Psychological symptoms include inability to concentrate, irritability and anxiety, poor memory, depressive mood and mood swings as well as lower interest in sexual activity [16, 24].

## **Long-Term Changes**

Most of physical and physiological changes caused by menopause usually resolve after this period. However, there are also long-term effects of climacteric, that include increased risk of osteopenia and osteoporosis, atherosclerosis and acute myocardial infarction as well as other cardiovascular diseases. The risk rises vehemently after menopause and may be reduced by controlling hypertension, overweight, increased blood lipids and tobacco smoking [25-27].

The age of menopause and intensity of menopause symptoms is an individual issue and may depend upon lifestyle factors and genes [28]. According to Bauld and Brown [29], emotional intelligence may also influence the intensity of menopause symptoms. It has been found that low emotional intelligence may be related to worse menopause symptoms, partly mediated by a negative attitude to menopause, high stress, anxiety and depression as well as low proactive coping. The association of menopause symptoms and HIV infection as well as drug-using has shown that HIV-uninfected women reported less intense menopause symptoms than those with HIV infection. Moreover, negative life events and depressive conditions are also highly associated with menopause symptoms intensity [30].

## **Factors Influencing the Appearance of Early Menopause**

Menopause is usually a natural, biological change in women body functioning. However, this process may occur earlier, for example in the

tobacco smokers. Everson et al. have found that both passive and active smoking may cause early menopause [31], whereas Cooper et al. [32] and Cramer et al. [33] have reported an association with active smoking, but not with passive smoking. It has been shown that stopping smoking more than ten years before menopause considerably reduced the risk of early menopause [33]. Moreover, the influence of other factors such as coffee and alcohol consumption has not on early menopause has no significant association [34].

Menopause may be also initiated by premature ovarian failure (POF), which is a condition characterized by infertility, sex steroid deficiency, amenorrhea as well as elevated gonadotropins [35]. POF affects 1% of women by 40 years of age and 0.1% by 30 years of age. It was believed that premature ovarian failure might be irreversible and was described as premature menopause. However, it young women suffering from premature ovarian failure are now known to have intermittent ovarian function [36, 37]. In the majority of women having POF, the underlying cause has not been identified. The already known causes include autoimmune ovarian damage involving anti-ovarian antibodies, which is associated with other autoimmune disorders. The antibodies have been reported in premature ovarian failure by several authors, however their specificity and pathogenic role are still questionable. Another cause of POF may be genetic aberrations. A large number of genes have been considered as potential factors causing POF; however, a few clear causal mutations have been identified. Other potential causes of premature ovarian failure include environmental factors (i.e., toxins and infections) as well as iatrogenic factors following chemo-, radiotherapeutic and surgical interventions as in malignancies [35-37]. However, in the majority of spontaneous cases of POF, the cause is unknown, i.e., it is generally idiopathic [37]. Premature ovarian failure may be diagnosed by high blood levels of follicle stimulating hormone (FSH) and luteinizing hormone (LH). This medical study must be performed at least at 3 occasions at least 4 weeks apart [37].

It should be underlined that women who struggle with some functional disorder affecting the reproductive system, such as cancer of the reproductive organs, endometriosis and polycystic ovary syndrome, may also have an early menopause, because functional disorders often significantly speed up the climacteric process [38]. Bilateral oophorectomy (removal of ovaries) is a common cause of early menopause because of the decrease in sex hormones (estrogen and progesterone) level in blood. Cessation of menses as a result of removal of the ovaries is called "surgical menopause." To investigate the physiological effect of removal of ovaries on women body, 35 female volunteers after bilateral oophorectomy were subjected to cognitive tests. Patients who had

a decline of estrogen level lower than 50% were observed to have more cognitive function downturn. Rapid decline in estrogen level as a result of surgical menopause was associated with a noxious effect on cognitive function [39].

## **HORMONE REPLACEMENT THERAPY (HRT)**

Hormone replacement therapy is any form of hormone therapy wherein the patient is given hormones. This kind of therapy is administered to supplement the deficiency of naturally occurring endogenous hormones, or to substitute other hormones for naturally occurring hormones [40]. HRT is frequently used for biologically natural menopause and is supposed to prevent discomfort caused by diminished circulating estrogen and progesterone hormones. This treatment is also recommended in the cases of the surgical or premature menopause [41]. The hormones involved in HRT are mainly estrogens, progesterone or progestins, and sometimes even testosterone. According to Nelson et al. [40] about 38% of postmenopausal women in the USA in 1995 used HRT to reduce symptoms of menopause (hot flashes, urogenital atrophy) and prevent from possible after-effects (cardiovascular disease, osteoporosis) of the deficiency of sex hormones. There are many advantages of hormonal replacement therapy. According to Scherwin [42], female patients who received estrogen, androgen or combination of these hormones had better results on cognitive tests (short-term and long-term memory, test of logical reasoning) in comparison to oophorectomized women who received placebo and had lower concentrations of plasma testosterone and estradiol. It is also assumed that estrogen might prevent from coronary heart disease, because it reduces low-density lipoprotein levels and elevates high-density lipoprotein levels [43, 44]. Moreover, it has been established that the prior hormone replacement therapy is associated with reduced risk of Alzheimer disease, however, there is no evident benefit with current use of this therapy unless this therapy lasts at least for ten years [45]. HRT is also applied to prevent hormone related skin ageing [46]. It has been proved by different scientific groups that estrogen therapy applied by women after menopause could delay collagen degradation and improve elastic properties of skin [47, 48].

Although HRT may bring many positive effects on women health, there are also a few doubts and harms connected with this therapy. According to Lacey et al. [49], female patients who used short-term replacement therapy containing only estrogen were not at increased risk, however long-term therapy (10 years

or more) was linked to a prominently increased risk of ovarian cancer. The possible association between the current use of oral HRT and increased risk of venous thromboembolism has also been investigated. It has been concluded that women aged 45–64 years using HRT are in the group of increased risk of VTE. The increased risk may be concentrated in new patients [50]. Another harms of HRT use may be also congenital heart disease, stroke, cholecystitis and breast cancer with 5 or more years of use [51]. There are many literature sources that report potential carcinogenic effects of hormonal replacement therapy onto breast tissue [e.g., 52-55]. According to Weiss et al. [56], continuous combined HRT may increase breast cancer risk among current, longer-term users and the risk can dissipate once use is discontinued. The results of other studies [57] have shown that long-term current use of progestin-estrogen combined HRT may increase the risk of breast cancer. However, the use of progestin-estrogen combination may reduce or avoid the risk of endometrial cancer that, by contrast, is elevated by the use of estrogen alone [57].

However, it remains disputable whether HRT stimulates breast cancer from scratch, or it may cause the growth of already existing small-size cancer nests, which would otherwise go undiagnosed [58].

## **Medicines Used in HRT**

There are many medicines containing hormones synthesized in a chemical reactions pathway. For example, birth control pills may be mentioned. These pills contain a combination of estrogen and progestin and may be used to relieve symptoms until menopause (not after menopause) and regulate menstrual bleeding [59]. To reduce one of the symptoms of menopause – the dryness of tissue in and around the vagina, cream, ring or tablets containing low-dose may be used [60]. Also pills and patches containing sex hormones in different concentrations and compositions may be used to release menopause symptoms [61]. Medicines containing estrogen are also used to release possible severe symptoms caused by sudden, early menopause as well as to prevent weakening bones [62]. It has been reported that a combination of testosterone and estrogen could be used to release symptoms of menopause that have not improved with estrogen therapy. According to the results of Goldstat et al. [63], testosterone therapy can improve sexual function and well-being mood in premenopausal women with low testosterone level and low libido. As a substantial number of women experience diminished sexual interest and well-being during their late reproductive years, further research is warranted to

evaluate the benefits and safety of longer-term intervention. However it must be underlined that this combination of sex hormones carries the same risks as estrogen treatment as well as testosterone risks and side effects [64]. It is very important to remember that HRT should be short-term and low-dose, moreover regular medical check-ups are required.

### **Contraindications for Supplementation with Synthetic Hormones**

Although hormonal replacement therapy may be helpful in reduction of symptoms of menopause, not every woman may use this kind of treatment. There are a few contraindications for supplementation with synthetic estrogen, including undiagnosed vaginal bleeding. As already mentioned, this symptom may indicate menopause as well as polyp or lesion as well as vaginal atrophy or functional endometrial response. Moreover, active liver diseases with abnormal liver function tests and suspected or active breast or endometrial cancer are also strong contraindications for HRT use. Some studies have indicated that the menopausal hormone therapy increases the risk of stroke and gallbladder disease [65]. Furthermore, this kind of therapy is not recommended for patients who suffer from acute-phase myocardial infarction and active thromboembolic disorder [50]. Therefore, the search for other possibilities to overcome problems associated with menopausal symptoms is continued.

### **Phytoestrogens as Alternative for Hormone Therapy Replacement**

A safer alternative to hormone replacement therapy could be supplements containing non-steroidal natural products known as phytoestrogens [66]. It has been proved that Asian women that consume high quantity of soybeans as a part of their diet are less affected by climacteric symptoms [67]. According to statistics, 70-80% women in western countries suffer from hot flushes and in China and Japan only 14-15% notify these problems [68, 69]. It is believed that food rich in phytoestrogens may prevent the postmenopausal problems. On the basis of this evidence, phytoestrogens have been studied as potential substances in HRT.

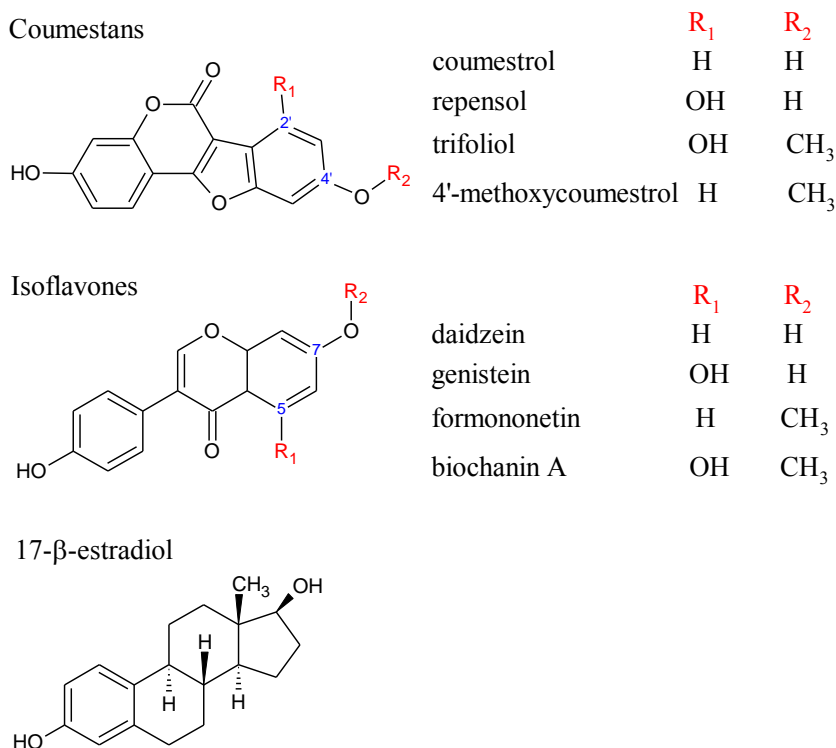


Figure 2. Coumestans and isoflavones as structural analogues of 17- $\beta$ -estradiol.

Phytoestrogens (PE) are chemicals produced by plants, which are structurally similar to steroidal estrogens (Figure 2). [70]. Phytoestrogens can be divided into different classes such as lignans, stilbenes, cumestans, chalcones and flavonoids [71]. However, isoflavones have been the most extensively studied phytoestrogens [72]. Additionally, they have been mostly applied in therapy for menopausal women. Isoflavones belong to the group known as flavonoids. The major isoflavones are genistein, daidzein and glycitin. They can be found in legumes such as soybean, red clover, chickpeas, peanuts, flaxseed, lentils and kidney beans [73]. So far most of the studies have been devoted to genistein and daidzein [74]. Plants contain their glycoside form, but during digestion the sugar moiety is cleaved and the aglycones of isoflavones are formed [75]. When the glucose residue is removed the isoflavones become active compounds. Phytoestrogens are able to bind to  $\alpha$  or  $\beta$  estrogen receptors, however with higher affinity to  $\beta$  subtype [76]. Phytoestrogens have similar

chemical structure to estrogens, therefore it is thought that PE could replace them in HRT.

## **Impact of Phytoestrogens on Menopausal Symptoms**

The phytoestrogen supplements applied by postmenopausal women are supposed to relieve climacteric symptoms such as hot flushes and night sweats. Different scientific groups examined the impact of phytoestrogen on these symptoms. Table 1 presents the results of published reviews and meta-analyses concerning the efficacy of phytoestrogens in reducing climacteric symptoms. Albertazzo and Purdie [77] have assessed the data from clinical trials of diets rich in soy and noticed a diminution in hot flushes of around 50% for diets containing 50-80 mg of isoflavones. In other studies it has been demonstrated that hot flashes and night sweats were reduced in women with higher intake of isoflavones [78, 79]. Jacobs et al. [80] have assessed the efficacy of isoflavone products in reducing the vasomotor symptoms in menopausal women. They concluded that there was no evidence, but only some indication that isoflavone supplements are beneficial in reducing hot flushes. Howes et al. [81] have made a systematic review to determine the efficacy of isoflavone supplementation, derived from red clover or soy, in minimizing the number of daily menopausal flushes. The results of meta-analysis favored isoflavones over placebo, however the difference was slight. Therefore, it was suggested that the isoflavone products might cause modest reduction in number of menopausal symptoms. However, beneficial effects may be noticeable by women experiencing a high number of flushes. Additionally, it was suggested that higher doses of phytoestrogens were more likely to ease the unfavorable symptoms. It has been also reported that the application of 54 mg of genistein reduced the hot flushes frequency after 1 year of treatment [82, 83]. Another scientific group has shown that the products containing more than 15 mg of genistein were more effective than the lower dose in reducing menopausal symptoms [84]. On the other hand, Leathaby et al. [85] have found no evidence that isoflavones were efficient in this area. Isoflavone supplements have been found relatively ineffective in reducing hot flushes, however isoflavone-rich foods exhibited beneficial effects better than placebo but still HRT seems to be more effective [86]. In another trial 21 postmenopausal women took placebo and 19 women received a supplement containing phytoestrogen. It was concluded that phytoestrogens connected with mixed exercise were not sufficient to reduce menopausal symptoms [87]. Nelson et al. [40] performed a systematic review regarding



dietary interventions and supplements to get contradictory results. It was concluded that there was no evidence that phytoestrogens can reduce the hot flushes.

It should be mentioned that most studies were performed over a short time and the dose was not clearly defined. Additionally, the absorption of phytoestrogens can be also different between individuals. Therefore it is difficult to compare the results and there is no conclusive evidence that phytoestrogens either as supplement or supplied in the diet have a significant effect on reduction of hot flushes frequency or severity.

**Table 1. Published reviews and meta-analyses presenting clinical studies of effectiveness of phytoestrogens in reducing menopausal symptoms**

Sample	Results	Ref
Phytoestrogens	Potential benefit	Glazier [88]
Phytoestrogens	Potential benefit	Ewies [89]
Isoflavones	Uncertain benefit	Phipps [90]
Phytoestrogens	Uncertain benefit	Jacquot [91]
<i>Actaearacemosa</i> extracts	Potential benefit	Viereck [92]
Isoflavones	Potential benefit	Howes [81]
Phytoestrogens	Uncertain benefit	Usui [93]
Soy isoflavones	No benefit	Hopper [94]
Isoflavones	Potential benefit	Williamson [95]

Hormone replacement therapy is also beneficial to treat skin aging in menopausal women. Mukrish et al. [96] proposed that the traditional Malaysian herb, known as *Labisiapumila* could be potential hormonal therapy towards skin aging. This plant is supposed to bring the same benefits as soy because it is known to contain phytoestrogens.

## Safety of Phytoestrogens

Tempfer et al. [97] have analyzed side effects observed in clinical trials. According to the results phytoestrogens had in general a safe side effect profile with slightly increased rates of gastrointestinal problems such as myalgia, abdominal pain and sleepiness. There was no evidence in trails concerning an increase in endometrial or breast cancer. The side effects were more often observed in women over age 55 than in younger woman. Additionally, no

cumulative dose effect with time was determined. Patisaul and Jefferson [98] have concluded in their review, that question if phytoestrogens are beneficial or harmful to human remains unresolved and the evidence for/against the health benefits and adverse effects of phytoestrogens may depend on age, health status, and even the presence or absence of specific gut microflora. Bedells et al. [99] have reported no link of phytoestrogens to an increase in breast cancer risk or increase in endometrial hyperplasia. Moreover, unlike HZT, the phytoestrogens may not increase blood clotting risk in postmenopausal women.

## CONCLUSION

Although the studies conducted so far in humans are limited, phytoestrogens can exhibit potential hormonal effects. Therefore, they can be still treated as alternative to HRT. The efficacy of phytoestrogens depends on many factors such as source and dose of supplement, duration of use, individual metabolism and general estrogenic state of the patient. Therefore, it is difficult to study the effectiveness of phytoestrogens. However, further clinical studies should be performed to confirm the potential health effects of these natural compounds to postmenopausal women.

## REFERENCES

- [1] O'Connor, A. M., Tugwell, P., Wells, G. A., Elmslie, T., Jolly, E., Hollingworth, G., McPherson, R., Bunn, H., Graham, I., Drake, E. (1998). A decision aid for women considering hormone therapy after menopause: decision support framework and evaluation. *Patient education and counseling*, 33(3), 267-279.
- [2] Ross, R. K., Paganini-Hill, A., Wan, P. C. and Pike, M. C. (2000). Effect of hormone replacement therapy on breast cancer risk: estrogen versus estrogen plus progestin. *Journal of the National Cancer Institute*, 92(4), 328-332.
- [3] Purdie, D. M., Bain, C. J., Siskind, V., Russell, P., Hacker, N. F., Ward, B. G., Quinn, M.A., Green, A. C. (1999). Hormone replacement therapy and risk of epithelial ovarian cancer. *British journal of cancer*, 81(3), 559.
- [4] Clarkson, T. B., Anthony, M. S., Williams, J. K., Honoré, E. K. and Cline, J. M. (1998). The potential of soybean phytoestrogens for postmenopausal

- hormone replacement therapy. *Experimental Biology and Medicine*, 217(3), 365-368.
- [5] *A primer for the perimenopausal*. The North American Menopause Society. Retrieved 11 April 2013.
- [6] McKinlay, S. M., Brambilla, D. J. and Posner, J. G. (1992). The normal menopause transition. *American Journal of Human Biology*, 4(1), 37-46.
- [7] Mckinlay, S. M. (1996). The normal menopause transition: an overview. *Maturitas*, 23(2), 137-145.
- [8] Federman, D. D. (2006). The biology of human sex differences. *New England Journal of Medicine*, 354(14), 1507-1514.
- [9] O'Connor, K. A., Holman, D. J. and Wood, J. W. (2001). Menstrual cycle variability and the perimenopause. *American Journal of Human Biology*, 13(4), 465-478.
- [10] Delaney, M. F. (2006). Strategies for the prevention and treatment of osteoporosis during early postmenopause. *American journal of obstetrics and gynecology*, 194(2), S12-S23.
- [11] Burger, H. G., Dudley, E. C., Robertson, D. M. and Dennerstein, L. (2001). Hormonal changes in the menopause transition. *Recent Progress in Hormone Research*, 57, 257-275.
- [12] Kumar, T. R., Wang, Y., Lu, N. and Matzuk, M. M. (1997). Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nature genetics*, 15(2), 201-204.
- [13] Danforth, D. R., Arbogast, L. K., Mroueh, J., Kim, M. H., Kennard, E. A., Seifer, D. B. and Friedman, C. I. (1998). Dimeric inhibin: a direct marker of ovarian aging. *Fertility and sterility*, 70(1), 119-123.
- [14] Maturana, M. A., Irigoyen, M. C. and Spritzer, P. M. (2007). Menopause, estrogens, and endothelial dysfunction: current concepts. *Clinics*, 62(1), 77-86.
- [15] Missmer, S. A., Eliassen, A. H., Barbieri, R. L. and Hankinson, S. E. (2004). Endogenous estrogen, androgen, and progesterone concentrations and breast cancer risk among postmenopausal women. *Journal of the National Cancer Institute*, 96(24), 1856-1865.
- [16] Hoffman, B., Schorge, J., Schaffer, J., Halvorson, L., Bradshaw, K. and Cunningham, F. (2012). *Williams gynecology*. McGraw Hill Professional.
- [17] Mencaglia, L., Perino, A. and Hamou, J. (1987). Hysteroscopy in perimenopausal and postmenopausal women with abnormal uterine bleeding. *The Journal of reproductive medicine*, 32(8), 577-582.
- [18] Anastasiadis, P. G., Koutlaki, N. G., Skaphida, P. G., Galazios, G. C., Tsikouras, P. N. and Liberis, V. A. (1999). Endometrial polyps:

- prevalence, detection, and malignant potential in women with abnormal uterine bleeding. *European journal of gynecological oncology*, 21(2), 180-183.
- [19] Goldstein, S. R., Nachtigall, M., Snyder, J. R. and Nachtigall, L. (1990). Endometrial assessment by vaginal ultrasonography before endometrial sampling in patients with postmenopausal bleeding. *American journal of obstetrics and gynecology*, 163(1), 119-123.
- [20] Bygdeman, M. and Swahn, M. L. (1996). Replens versus dienoestrol cream in the symptomatic treatment of vaginal atrophy in postmenopausal women. *Maturitas*, 23(3), 259-263.
- [21] Monterrosa-Castro, A., Romero-Pérez, I., Marrugo-Flórez, M., Fernández-Alonso, A. M., Chedraui, P. and Pérez-López, F. R. (2012). Quality of life in a large cohort of mid-aged Colombian women assessed using the Cervantes Scale. *Menopause*, 19(8), 924-930.
- [22] Chedraui, P., Pérez-López, F. R., Mendoza, M., Leimberg, M. L., Martínez, M. A., Vallarino, V. and Hidalgo, L. (2010). Factors related to increased daytime sleepiness during the menopausal transition as evaluated by the Epworth sleepiness scale. *Maturitas*, 65(1), 75-80.
- [23] Arakane, M., Castillo, C., Rosero, M. F., Peñafiel, R., Pérez-López, F. R. and Chedraui, P. (2011). Factors relating to insomnia during the menopausal transition as evaluated by the Insomnia Severity Index. *Maturitas*, 69(2), 157-161.
- [24] Llaneza, P., García-Portilla, M. P., Llaneza-Suárez, D., Armott, B. and Pérez-López, F. R. (2012). Depressive disorders and the menopause transition. *Maturitas*, 71(2), 120-130.
- [25] Mitchell, R. S., Kumar, V., Abbas, A. K. and Fausto, N. (2007). Robbins basic pathology. *Philadelphia: Saunders*, 8, 345-355.
- [26] Souza, H. C. and Tezini, G. C. (2014). Autonomic cardiovascular damage during post-menopause: the role of physical training. *Aging and disease*, 4(6), 320-328.
- [27] ESHRE Capri Workshop Group. (2011). Perimenopausal risk factors and future health. *Human reproduction update*, 17(5), 706-717.
- [28] De Bruin, J. P., Bovenhuis, H., Van Noord, P. A. H., Pearson, P. L., Van Arendonk, J. A. M., TeVelde, E. R., Kuurman, W.W., Dorland, M. (2001). The role of genetic factors in age at natural menopause. *Human Reproduction*, 16(9), 2014-2018.
- [29] Bauld, R. and Brown, R. F. (2009). Stress, psychological distress, psychosocial factors, menopause symptoms and physical health in women. *Maturitas*, 62(2), 160-165.

- [30] Miller, S. A., Santoro, N., Lo, Y., Howard, A. A., Arnsten, J. H., Floris-Moore, M., Moskaleva, G., Schoenbaum, E. E. (2005). Menopause symptoms in HIV-infected and drug-using women. *Menopause*, 12(3), 348-356.
- [31] Everson, R. B., Sandler, D. P., Wilcox, A. J., Schreinemachers, D., Shore, D. L. and Weinberg, C. (1986). Effect of passive exposure to smoking on age at natural menopause. *British medical journal (Clinical research ed.)*, 293(6550), 792.
- [32] Cooper, G. S., Sandler, D. P. and Bohlig, M. (1999). Active and passive smoking and the occurrence of natural menopause. *Epidemiology*, 10(6), 771-773.
- [33] Cramer, D. W., Harlow, B. L., Xu, H., Fraer, C. and Barbieri, R. (1995). Cross-sectional and case-controlled analyses of the association between smoking and early menopause. *Maturitas*, 22(2), 79-87.
- [34] Mikkelsen, T. F., Graff-Iversen, S., Sundby, J. and Bjertness, E. (2007). Early menopause, association with tobacco smoking, coffee consumption and other lifestyle factors: a cross-sectional study. *BMC Public Health*, 7(1), 1.
- [35] Goswami, D. and Conway, G. S. (2005). Premature ovarian failure. *Human reproduction update*, 11(4), 391-410.
- [36] Beck-Peccoz, P. and Persani, L. (2006). Premature ovarian failure. *Orphanet Journal of Rare Diseases*, 1(1), 1.
- [37] Kalantaridou, S. N., Davis, S. R. and Nelson, L. M. (1998). Premature ovarian failure. *Endocrinology and metabolism clinics of North America*, 27(4), 989-1006.
- [38] Harlow, B. L. and Signorello, L. B. (2000). Factors associated with early menopause. *Maturitas*, 35(1), 3-9.
- [39] Farrag, A. K. F., Khedr, E. M., Abdel-Aleem, H. and Rageh, T. A. (2002). Effect of surgical menopause on cognitive functions. *Dementia and geriatric cognitive disorders*, 13(3), 193-198.
- [40] Nelson, H. D., Humphrey, L. L., Nygren, P., Teutsch, S. M. and Allan, J. D. (2002). Postmenopausal hormone replacement therapy: scientific review. *Jama*, 288(7), 872-881.
- [41] Shuster, L. T., Rhodes, D. J., Gostout, B. S., Grossardt, B. R. and Rocca, W. A. (2010). Premature menopause or early menopause: long-term health consequences. *Maturitas*, 65(2), 161-166.
- [42] Sherwin, B. B. (1988). Estrogen and/or androgen replacement therapy and cognitive functioning in surgically menopausal women. *Psychoneuroendocrinology*, 13(4), 345-357.

- 
- [43] Stampfer, M. J. and Colditz, G. A. (1991). Estrogen replacement therapy and coronary heart disease: a quantitative assessment of the epidemiologic evidence. *Preventive medicine*, 20(1), 47-63.
- [44] Nabulsi, A. A., Folsom, A. R., White, A., Patsch, W., Heiss, G., Wu, K. K. and Szklo, M. (1993). Association of hormone-replacement therapy with various cardiovascular risk factors in postmenopausal women. *New England Journal of Medicine*, 328(15), 1069-1075.
- [45] Zandi, P. P., Carlson, M. C., Plassman, B. L., Welsh-Bohmer, K. A., Mayer, L. S., Steffens, D. C., Breitner, J. C., Cache County Memory Study Investigators. (2002). Hormone replacement therapy and incidence of Alzheimer disease in older women: the Cache County Study. *Jama*, 288(17), 2123-2129.
- [46] Schmid, D., Hanay C., Muggli, R., Züllli, F. (2001) Genistein, a new cosmetic ingredient derived from soy. *SÖFW-Journal*, 127, 10, 22-27.
- [47] Dunn, L.B., Damesyn, M., Moore, A.A., Reuben, D.B., Greendale, G.A. (1997) Does estrogen prevent skin ageing? *Archives of Dermatology* 133, 339-342.
- [48] Callens, A., Vaillant, L., Lecomte, P., Berson, M., Gall, Y., G. Lorette, G. (1996) Does hormonal skin Ageing exist? A study of the influence of different hormone therapy regimes on the skin of postmenopausal women using non-invasive measurement techniques. *Dermatology* 193, 289-294.
- [49] Lacey Jr, J. V., Mink, P. J., Lubin, J. H., Sherman, M. E., Troisi, R., Hartge, P., Schatzkin, A., Schairer, C. (2002). Menopausal hormone replacement therapy and risk of ovarian cancer. *Jama*, 288(3), 334-341.
- [50] Daly, E., Vessey, M. P., Hawkins, M. M., Carson, J. L., Gough, P. and Marsh, S. (1996). Risk of venous thromboembolism in users of hormone replacement therapy. *The Lancet*, 348(9033), 977-980.
- [51] US Preventive Services Task Force (2005). Hormone therapy for the prevention of chronic conditions in postmenopausal women: recommendations from the US Preventive Services Task Force. *Annals of Internal Medicine* 142(10), 855.
- [52] Beral, V., Banks, E., Reeves, G. and Appleby, P. (1998). Use of HRT and the subsequent risk of cancer. *Journal of epidemiology and biostatistics* 4(3), 191-210.
- [53] Chen, C. L., Weiss, N. S., Newcomb, P., Barlow, W. and White, E. (2002). Hormone replacement therapy in relation to breast cancer. *Jama* 287(6), 734-741.

- [54] Kumle, M. (2008). Declining breast cancer incidence and decreased HRT use. *The Lancet*, 372(9639), 608-610.
- [55] Wile, A. G., Opfell, R. W. and Margileth, D. A. (1993). Hormone replacement therapy in previously treated breast cancer patients. *The American journal of surgery* 165(3), 372-375.
- [56] Weiss, L. K., Burkman, R. T., Cushing-Haugen, K. L., Voigt, L. F., Simon, M. S., Daling, J. R., Norman, S.A., Bernstein, L., Ursin, G., Marchbanks, P.A., Strom, B.L., Berlin, J.A., Weber, A.L., Doody, D.R., Wingo, P.A., McDonald, J.A., Malone, K.E., Folger, S.G., Spirtas, R. (2002). Hormone replacement therapy regimens and breast cancer risk. *Obstetrics and Gynecology* 100(6), 1148-1158.
- [57] Persson, I., Weiderpass, E., Bergkvist, L., Bergström, R. and Schairer, C. (1999). Risks of breast and endometrial cancer after estrogen and estrogen-progestin replacement. *Cancer Causes and Control*, 10(4), 253-260.
- [58] Dietel, M. (2010). Hormone replacement therapy (HRT), breast cancer and tumor pathology. *Maturitas* 65(3), 183-189.
- [59] Miller, L. and Hughes, J. P. (2003). Continuous combination oral contraceptive pills to eliminate withdrawal bleeding: a randomized trial. *Obstetrics and Gynecology* 101(4), 653-661.
- [60] Speroff, L. and Fritz, M. A. (Eds.). (2005). *Clinical gynecologic endocrinology and infertility*. Lippincott Williams and Wilkins.
- [61] Schmidt, P. (2012). The 2012 hormone therapy position statement of the North American Menopause Society. *Menopause (New York, NY)*, 19(3), 257.
- [62] North American Menopause Society. (2010). Estrogen and progestogen use in postmenopausal women: 2010 position statement of The North American Menopause Society. *Menopause (New York, NY)*, 17(2), 242.
- [63] Goldstat, R., Briganti, E., Tran, J., Wolfe, R. and Davis, S. R. (2003). Transdermal testosterone therapy improves well-being, mood, and sexual function in premenopausal women. *Menopause* 10(5), 390-398.
- [64] North American Menopause Society. (2005). The role of testosterone therapy in postmenopausal women: position statement of The North American Menopause Society. *Menopause (New York, NY)*, 12(5), 496.
- [65] Farquhar, C.M., Marjoribanks, J., Lethaby, A., Lamberts, Q., Suckling, J.A. (2005). Long term hormone therapy for perimenopausal and postmenopausal women. *Cochrane Database Sys Rev* 2005, CD004143.
- [66] Poluzzi, E., Piccinni, C., Raschi, E., Rampa, A., Recanatini, M., De Ponti, F. (2014). Phytoestrogens in postmenopausal women: the state of the art. From a

- chemical, pharmacological and regulatory perspective. *Current Medicinal Chemistry* 21, 417-436.
- [67] Aldercreutz, H., Hamalainen, E., Gorbach, S., Goldin, B. (1992). Dietary phyto-estrogen and the menopause in Japan. *Lancet* 339, 1233.
- [68] Tan, G.W. (1994). The climacteric of Chinese factory workers. *Maturitas* 19, 177-82.
- [69] Guthrie, J., Dennerstein, L., Hopper, J., Burger, H.G. (1996). Hot flushes, menstrual status and hormone levels in a population based sample of midlife women. *Obstetrics and Gynecology* 88, 437-442.
- [70] Knight, D.C., Eden, J.A. (1996). A review of the clinical effects of phytoestrogens. *Obstetrics and Gynecology* 87, 897-904.
- [71] Sirotkin, A.V., Harrath, A.H. (2014). Phytoestrogens and their effects. *European Journal of Pharmacology* 741, 230-238.
- [72] Baber, R. (2010). Phytoestrogens and post reproductive health. *Maturitas* 66, 322-349.
- [73] Vacek, J., Klejdus, B., Lojkova, L., Kuban, V. (2008). Current trends in isolation, separation, determination and identification of isoflavones: a review. *Journal of Separation Science* 31, 2054-67.
- [74] Dixon, R.A. (2004) Phytoestrogens, *The Annual Review of Plant Biology* 55, 225–261.
- [75] Hulem, R., Blair, R.M. (2006). Soy isoflavones for postmenopausal symptoms. An examination of evidence. *Advance for nursepractitioners* 14, 32-38.
- [76] Barone, M., Tanzi, S., Lofano, K., Scavo, M.P., Guido, R., Demarinis, L., Principi, M.B., Bucci, A., Di Leo, A. (2008). Estrogens, phytoestrogens and colorectal neoproliferative lesions. *Genes and Nutrition* 3(1), 7–13.
- [77] Albertazii, P., Purdie, D.W. (2002). The nature and utility of phytoestrogens. A review of the evidence. *Maturitas* 42, 172-185.
- [78] Taku, K., Melby, M.K., Kronenberg, F., Kurzner, M.S., Messina, M. (2012) Extracted or synthesized soybean isoflavones reduce menopausal hot flash frequency and severity: systematic review and meta-analysis of randomized controlled trials. *Menopause* 19(7), 776-90.
- [79] Bolanos-Diaz, R., Zavala-Gonzales, J.C., Mezones-Holguin, E., Francia-Romero, J. (2011). Soy extracts versus hormone therapy for reduction of menopausal hot flushes: indirect comparison *Menopause* 18(7) 825–829.
- [80] Jacobs, A., Wegewitz, U., Sommerfeld C., Grossklaus, R., Lampen, A., (2009) Efficacy of isoflavones in relieving vasomotor menopausal symptoms- A systematic review. *Molecular Nutrition and Food Research* 53,1084-1097.



- [81] Howes, L.G., Howes, J.B., Knight, D.C. (2006). Isoflavone therapy for menopausal flushes: A systematic review and meta-analysis. *Maturitas* 55, 203-211.
- [82] Crisafulli, A., Marini, H., Bitoo, A., Altavilla, D., Squadrito, G., Romeo, A., Adamo, E.B., Marini, R., D'Anna, R., Corrado, F., Bartolone, S., Frisina, N., Squadrito, F. (2004). *Menopausue* 11(4) 400-404.
- [83] D'Anna R, Cannata M, AtteritanoM, Cancellieri, F., Corrado, F., Baviera, G., Triolo, O., Antico, F., Gaudio, A., Frisina, N., Bitto, A., Polito, F., Minutoli, L., Altavilla, D., Marini, H., Squadrito, F. (2007). Effects of the phytoestrogen genestein on hot flushes, endometrium and vaginal epithelium in post- menopausal women. A 1 year randomized double blind placebo controlled study. *Menopause* 14, 648–55.
- [84] Kurzer, M. (2008) Soy consumption for reduction of menopausal symptoms. *Inflammopharmacology* 16, 227–9.
- [85] Leathaby, A.E., Brown, J., Marjoribanks, J., Kronenberg, F., Roberts, H., Eden, J. (2007). Phytoestrogens for vasomotor menopausal symptoms. *Cochrane Database Sys Rev.* 17, 4, CD001395.
- [86] Cassidy, A. (2004). Phytoestrogens and women's health. *Women's health medicine* 1(1) 30-33.
- [87] Riesco, E., Choquette, S., Audet, M., Tessier, D., Dionne, I.J. (2011). Effect of exercise combined with phytoestrogens on quality of life in postmenopausal women. *Climacteric: The Journal of the International Menopause Society* 14(5) 573–580.
- [88] Glazier, M.G., Bowman, M.A. (2001). A review of the evidence for the use of phytoestrogens as a replacement for traditional estrogen replacement therapy. *Archives of Internal Medicine* 161(9) 1161-1172.
- [89] Ewies, A.A. (2002). Phytoestrogens in the management of the menopause: up-to-date. *Obstetrical and Gynecological Survey* 57(5) 306-313.
- [90] Phipps, W.R., Duncan, A.M., Kurzer, M.S. (2002). Isoflavones and postmenopausal women: a critical review. *Treatments in endocrinology* 1(5) 293-311.
- [91] Jacquot, Y., Rojas, C., Refouvet, B., Robert, J.F., Leclercq, G., Xicluna, A. (2003). Recent advances in the development of phytoestrogens and derivatives: an update of the promising perspectives in the prevention of postmenopausal diseases. *Mini-Reviews in Medicinal Chemistry* 3(5) 387-400.
- [92] Viereck, V., Emons, G., Wuttke, W. (2005) Black cohosh: just another phytoestrogen? *Trends in Endocrinology and Metabolism* 16(5) 214-221.

- 
- [93] Usui, T. (2006). Pharmaceutical prospects of phytoestrogens. *Endocrine Journal* 53(1) 7-20.
- [94] Hooper, L., Madhavan, G., Tice, J.A., Leinster, S.J., Cassidy, A. (2010). Effects of isoflavones on breast density in pre- and post-menopausal women: a systematic review and meta-analysis of randomized controlled trials. *Human Reproduction Update* 16(6) 745-760.
- [95] Williamson, G., Coppens, P., Serra-Majem, L., Dew, T. (2011). Review of the efficacy of green tea, isoflavones and aloe vera supplements based on randomised controlled trials. *Food and Function* 2(12) 753-859.
- [96] Mukrish, M.H., Akhir, N.A.M., Majid, F.A.A., Yaakob, H., Azila, A.A., Sarmidi, M.R. (2013). Phytoestrogen in skin ageing: The case of *LabisiaPumila*. *Regenerative Research* 2(1) 7-13.
- [97] Tempfer, C.B., Froese, G., Heinze, G., Bentz, E.K., Hefler, L.A., Huber, J.C. (2009). Side effects of phytoestrogens: a meta- analysis of randomized trials. *The American Journal of Medicine* 122, 939–946.
- [98] Patisaul, H. B. and Jefferson, W. (2010). The pros and cons of phytoestrogens. *Frontiers in Neuroendocrinology* 31(4) 400-419.
- [99] Bedell, S., Nachtigall, M. and Naftolin, F. (2014). The pros and cons of plant estrogens for menopause. *The Journal of Steroid Biochemistry and Molecular Biology* 139, 225-236.



*Chapter 8*

## **PREVENTIVE MEASURES OF DEVELOPING OSTEONECROSIS OF THE JAW IN PATIENTS RECEIVING BISPHOSPHONATE THERAPY**

***Vladimíra Schwartzová, MD,<sup>1</sup> Peter Kizek, MD,<sup>1</sup> and  
Jozef Minčík, MD.<sup>2</sup>***

<sup>1</sup>I. Department of Stomatology Pavol Jozef Šafárik University, Faculty of  
Medicine Košice and University Hospital LP Košice, Slovakia

<sup>2</sup>Private clinic Košice, Slovakia

Corresponding Author: vladka.schwartzova@gmail.com

### **ABSTRACT**

Osteonecrosis of the jaw is a rare, but very severe complication in patients receiving bisphosphonate therapy. This complication significantly restricts food intake and reduces their quality of life. The negative influence of bisphosphonates on jaw bones is still not precisely known and is the subject of research.

The authors present the increasing number of negative effects of bisphosphonates when used orally or parenterally. Treatment is protracted and very complicated. The authors present clinical symptoms and treatment options based on their own results, while highlighting the most interesting cases from the treatment group.

In the final part of the article, the authors emphasize the significance of preventive measures. In accordance with other studies, the best way to

prevent osteonecrosis of the jaw is to observe set precautions (especially before the bisphosphonate treatment initiates).

**Keywords:** bisphosphonates, osteonecrosis of the jaw, BRONJ, osteonecrosis prevention

## INTRODUCTION

Bisphosphonates are often prescribed as highly effective preparations which prevent bone loss in many diseases characterized by increased bone resorption, such as senile osteoporosis in men and women, Paget's disease, glucocorticoids-induced osteoporosis [4, 13], multiple myeloma, bone metastases of breast, lung, or prostate carcinoma [14]. They benefit the patient by slowing the resorption of bone mass and reducing bone pain. The restriction of bone resorption also guards against the dangerous, malignant, disease-induced hypercalcemia [1].

Bisphosphonates certainly contribute to improving the quality of life.

Even though they are well tolerated by the patients, the use of bisphosphonates may be limited for some patients due to its adverse effects.

Each complication or every necessary intervention during the therapy reduces the patient's quality of life and may worsen their prognosis.

Currently, most of the bisphosphonate related osteonecroses form as a result of a breach of integrity of the mucous membranes after an invasive procedure in the oral cavity and exposing the alveolar bone, without simultaneous implementation of measures to prevent drug chemonecrosis. The cause of this condition can be attributed to the lack of communication between the medical practitioner prescribing high-risk drugs, the patient and the dentist, and the lack of awareness about the issue in both patients and dentists. The doctor who prescribes a problematic drug is obligated to inform the patient about the risks and adverse effects of the planned treatment and to remind them to specifically inform their dentist about this fact. When the specialist physician neglects this obligation, the patient is left uninformed about the incurred risks.

## THE ADVERSE EFFECTS OF BISPHOSPHONATES

Some of the adverse effects of bisphosphonates were recognized long ago, such as irritated esophagus mucous membrane. More serious complications of

this treatment were identified only recently, such as osteonecrosis of the jaw or bone turnover suppression, reflecting the relatively rare occurrence of such adverse effects.

Short-term adverse effects of bisphosphonates include gastrointestinal upset, flu-like syndrome acute phase reaction (flu symptoms) after bisphosphonate administration [5], severe musculoskeletal pain, hypocalcemia, esophageal carcinoma due to orally administered bisphosphonates or ocular tissue inflammation. Long-term effects include osteonecrosis of the jaw, atrial fibrillation, excessive bone turnover suppression or subtrochanteric fractures.

The issue of adverse effects of bisphosphonates is the subject of several debates. On the one hand, there is a proven positive effect in cancer and osteoporotic patients, but on the other hand the bisphosphonate therapy is increasingly associated with adverse effects on the jaw, such as necrosis of alveolar processes. Their emergence is probably multifactorial and will be the subject of further investigation and evaluation of patients' files [8].

## **BISPHOSPHONATE-ASSOCIATED OSTEONECROSIS OF THE JAW (BON)**

Shortly after the introduction of highly efficient nitrogenous types, mainly in treatment of cancer, the adverse effects manifested themselves in an unusual complication – osteonecrosis of the jaw.

Bisphosphonate-associated osteonecrosis of the jaw – BON was first described in 2003 [12]. The American Association of Oral and Maxillofacial Surgeons (AAOMS) uses the name Bisphosphonate-related osteonecrosis of the jaw – BRONJ [3]. Bisphosphonate-associated osteonecrosis of the jaw is not an entirely new phenomenon. A similar illness was described in the first half of the 19<sup>th</sup> century concerning match factory workers with so-called “phossy jaw” [7]. The detailed mechanism of this osteonecrosis is still being examined.

According to AAOMS [3], the estimated incidence is different in patients receiving intravenous bisphosphonates than those taking oral bisphosphonates. Based on the studies, the incidence in intravenous bisphosphonates ranges from 0.8% to 12%, while the risk in patients with orally administered bisphosphonates is lower, ranging from 0.01% to 0.04%. However, the incidence is higher after the extraction of teeth – from 0.09% to 0.34%.

Currently, the estimated incidence of osteonecrosis of the jaw in patients with orally administered bisphosphonates is approximately 1/10 000 to 1/100

000, although this estimate is largely based on incomplete data. The incidence of BON in cancer patients, who usually used high doses of intravenous bisphosphonates in more frequent intervals than patients with other diseases, is estimated to be 1 to 10 cases in 100 patients [9].

From our experience, we can confirm more frequent occurrence of osteonecrosis of the jaw, ranging from approximately 1 to 10 percent, in patients with intravenously administered medicinal products (zoledronate, pamidronate) and around 1% with tablets. Mandible (68%) tends to be affected more frequently than maxilla (28%). Both tend to be affected rarely (4%) [20].

Bisphosphonate-associated osteonecrosis of the jaw (BON) is an affection that must meet three criteria [3]:

1. The patient is, or has been treated with bisphosphonate.
2. An exposed, necrotic bone is present for at least 8 weeks (2-3 months) in the maxillofacial area.
3. The patient has never undergone a radiotherapy in maxillofacial area.

The risk factors that we believe increase the risk of developing BON are (Table 1):

- Invasive preservation or surgical operation in the oral cavity while taking bisphosphonates.
- Herpes or another infection in the area.
- Long-term use of corticosteroids.
- Medications that suppress the immune system.
- Diabetes mellitus.
- Blood clotting or blood flow disorder.
- Condition after hematopoietic stem cells transplant.
- Alcoholism, smoking, malnutrition.
- Overall bad condition.
- Osteopenia/Osteoporosis.
- Metastases in jaw bones.
- Untreated dentition and periodontium, lack of dental hygiene.
- Unsuitable prosthesis.

On the basis of the presented results of studies and according to the method and time interval of administration of bisphosphonate we divide patients into certain risk groups. Table 2 shows risk profiles in antiresorptive therapy [21].

**Table 1. Risk profiles for antiresorptive treatment**

Low risk	0.1-0.5%	bisphosphonates orally for less than three years, during the primary osteoporosis
Medium risk	1-5%	i.v. bisphosphonates in a twice-yearly and yearly intervals with secondary osteoporosis denosumab in monthly intervals in a dose of 120 mg
High Risk	4-20%	i.v. bisphosphonates monthly with cancer indications

**Table 2. Factors promoting individual risk of developing chemonecrosis**

High-risk medications	<ul style="list-style-type: none"> <li>• bisphosphonates containing nitrogen (II. and III. generation)</li> <li>• i.v. bisphosphonates in monthly intervals</li> <li>• orally administered bisphosphonates for longer than three years</li> <li>• combinations of high-risk medicaments</li> </ul>
Co-morbidity	<ul style="list-style-type: none"> <li>• diabetes mellitus</li> <li>• various immunodeficient conditions</li> <li>• chronic infections</li> <li>• impaired liver and kidney function</li> </ul>
Concurrent therapy	<ul style="list-style-type: none"> <li>• chronic treatment with steroids</li> <li>• chemotherapy</li> </ul>
Local factors	<ul style="list-style-type: none"> <li>• bad oral hygiene condition</li> <li>• pre-existing oral cavity diseases</li> </ul>
Abuse	<ul style="list-style-type: none"> <li>• smoking</li> <li>• alcohol</li> </ul>





Figure 1. Osteonecrotic jaw lesion. At least two-month-old, unhealed extraction wound in a patient receiving bisphosphonates/department collection.

## **CLINICAL PICTURE OF BISPHOSPHONATE-ASSOCIATED OSTEONECROSIS OF THE JAW**

Osteonecrosis of the jaw manifests itself with a non-healing wound after a tooth extraction, painful tooth socket with exposed necrotic bone, bad breath, possible signs of inflammation in the area, purulent discharge from the wound or the findings on an x-ray [17].

Figure 1 shows the extraction wound in a patient treated with bisphosphonates, which have not healed in more than two months.

We divide osteonecrosis clinically into three stages (Table 3) [15]. High-risk patients take bisphosphonate, in addition to having problems with swelling of soft tissues and vague soreness even before the clinical manifestation of osteonecrosis.

Bone condensation in the area around the unhealed extraction wound is present in the x-ray in the early stages. Later, osteolysis and sequestra formation begin. The bone defect is accompanied by irritation and inflammation of the surrounding soft tissues. Ongoing osteonecrosis can cause loose teeth. Advanced stages lead to pathological fractures (Figure 2), extraoral fistulas (Figure 3), or the emergence of oronasal or oroantral communication.

**Table 3. Clinical stages of osteonecrosis [15]**

The risk of an expanding osteonecrosis	Patients taking bisphosphonates without the presence of a lesion on an exposed or a necrotic bone (swelling of soft tissues and vague pain may be present before the clinical manifestation)
Stage I.	Asymptomatic lesion on an exposed or a necrotic bone with no sign of infection
Stage II.	Exposed or necrotic bone with inflammatory symptoms (pain, erythema, with or without purulent exudation)
Stage III.	Symptoms similar to stage II. and at least one of the following: pathological fracture, extraoral fistula, osteolysis reaching ramus of the mandible, oronasal or orotracheal communication



Figure 2. The third stage of bisphosphonate-related osteonecrosis of the jaw. Pathological fracture on the left side of the mandible body in a patient treated with bisphosphonate (department collection).

## THE FORMATION OF OSTEONECROSIS OF THE JAW

Several authors reported that the disruption of alveolar bone and mucosal cover after tooth extraction contribute to the formation of osteonecrosis of the jaw. It is also important to remember that this osteonecrosis may arise

spontaneously in predilectory areas such as mandibular and palatal tori or linea mylohyoidea, for example due to improperly fabricated prosthesis. The bone wound healing is disrupted and very difficult due to the effects of bisphosphonates. The anti-angiogenic effect of bisphosphonates (lack of blood supply) then leads to an expanding necrosis. The risk of developing BON increases with the length of the application and with the chosen dosage of bisphosphonates. Most BON patients used some of the bisphosphonates for more than three years, while the majority of them intravenously, but some also orally [3]. Intravenously for more than a year [20]. The cases of BON in patients using bisphosphonates orally are starting to appear in literature more often [18]. Bisphosphonates remain very firmly bonded and active in bones for a number of years [3]. So far, there is no known method of estimating potential risk of developing BON for a particular patient. Not even examination of serum CTX peptide concentration (carboxyterminal telopeptide of type I collagen) can be recommended from the available data to assess the risks of developing osteonecrosis of the jaw [9].

The infection in the socket plays an unclear role in etiopathogenesis. Often described is a colonization by fibrous, actinomyces-like organisms with tendencies to form biofilm and increase adherence for other microorganisms in the oral cavity [10, 11]. Despite frequent occurrence of these bacteria conglomerates in patients with BON, it has still not been proven whether an infection plays a primary, or secondary role in pathophysiology of BON [2]. However, infection certainly plays an important role at the time of osteonecrosis treatment. Actinomycosis should also be searched for microbiologically, because standard aerobic cultivation cannot capture actinomycetes. These bacteria are anaerobic, or rather microaerophilic (require reduced air pressure), non-sporulating microorganisms that are necessary to be cultivated under the anaerobic conditions. If species of *Actinomyces* are found, the scheme of administered antibiotics should be altered [16].

## **BON THERAPY**

Therapy of an already emerged BON can be lengthy and difficult. It is recommend to rinse the areas with antiseptic solutions containing chlorhexidine during the early stages of the illness. Moreover, meticulous oral hygiene, treatment of oral cavity or resolving any possible irritations of mucous membranes by overhanging fillings or inappropriate prosthetic jobs are also recommended. The more advanced stages need to be addressed surgically

according to the scope of the problem (equalisation, sequestrectomy, partial mandibulectomy). Surgical therapy can be very difficult due to poor healing of the damaged tissue.

In case of surgical intervention, antibiotics should be prescribed as a prophylaxis of secondary infections. The most commonly recommended antibiotics for BON prevention are clavulanate-potentiated amoxicillins, such as *Amoksiklav* or *Augmentin*, 625 mg orally, every 8 hours for 7 to 10 days, or 2 x 1 g at least 2 hours before surgery and 10-14 days after the surgery. When an allergy to PCN is known, doxycycline, e.g., *Deoxymykoin* one 100 mg tablet orally daily for 7 to 10 days should be prescribed. When long term administration is needed, we prescribe low toxic lincosamide antibiotics, such as *Dalacin C* 300 mg orally every 8 hours for 2 to 3 weeks.

## BON PREVENTION

A number of recommendations for doctors, dentists or maxillofacial surgeons were made to reduce the risk of developing BON. Recommendations were made by Slovak Oncology [19] and Myeloma Societies [20].



Figure 3. The third stage of bisphosphonate-related osteonecrosis of the jaw. Two extraoral fistulas in submental and submandibular region (department collection).

## **THE PROCEDURES IN PATIENTS BEFORE THE SCHEDULED INTRODUCTION OF HIGH-RISK DRUGS**

The optimal approach to prevent chemonecrosis of the jaw is to realize a complete dental rehabilitation before the introduction of high-risk drugs.

The point is to remove all of the potential entry points and sources of oral cavity infection and pathological conditions which would potentially require invasive surgical rehabilitation in the near future. The radicality of the treatment depends on individual risk of particular patient (Table 2). The patients with high risk of developing chemonecrosis should be treated more radically than the patients with lower risk. In this respect, the specialist doctor who decides whether to treat with high-risk medications or not bears crucial responsibility. Prior to their scheduled deployment, the doctor should send the patient to a mandatory dental examination and subsequently to any necessary restoration in order to avoid the need for invasive interventions in the oral cavity for several years. The therapy can only be deployed after comprehensive treatment by a dentist, while the dentist must check the patient every half a year to check the onset of possible spontaneous formation of chemonecrosis.

## **PROCEDURE FOR PATIENTS TREATED WITH HIGH-RISK MEDICATIONS**

Patients taking high-risk drugs need to be regularly checked at least once every six months in order to maintain an optimal state of oral hygiene, or to possibly reveal a spontaneously emerged chemonecrosis. Invasive methods should be avoided as much as possible when treating such patients, preferably using less invasive methods of treatment (e.g., preferred endodontic examination rather than extraction).

In case that it is necessary to implement an invasive procedure, it is necessary to follow the recommended protocol in order to prevent the emergence of chemonecrosis and to monitor the healing process until fully healed.

Each invasive procedure in the oral cavity of the patient treated by bisphosphonates must be consulted with a specialist (rheumatologist, orthopedist, hematologist/oncologist) and must be safeguarded by administering prophylactic antibiotics.

Bisphosphonates in human body do not metabolize. The ones that have not bound to the bone tissue are expelled from the organism by urine. Bisphosphonates bound in bone tissue release gradually due to osteoclast activity during bone remodeling, while only a part is expelled from the organism and the rest gets bound again (re-uptake). Thus, the patient receiving bisphosphonate therapy remains at risk even after discontinuation of the treatment and the drugs. For this reason, a short-term discontinuation of bisphosphonates has no substantial effect on the prognosis of chemonecrosis treatment.

It is recommended to suspend the application of bisphosphonates for several months. With regard to the risk of progression of the underlying disease, as well as the patient's psychological state, it is always a very difficult decision. In the past, the prevailing opinion was to suspend the treatment for a minimum of six months [3], or, according to Slovak Myeloma Society, two months before the extraction and then restart the treatment 2 months after that if the wound is fully healed [20].

At present, according to AAOMS's recommendations from 2009 [16], to reduce the risk of developing BON, the doctor can decide to interrupt the oral bisphosphonate treatment three months before and continue with it three months after a dental surgery. Suspending intravenously administered bisphosphonates does not provide any short-term benefits. Yet, if the status of the underlying disease permits long-term suspension, it may be beneficial in reducing the occurrence of new deposits or debilitating the clinical symptoms of BON [6, 16]. To confirm or refute the effectiveness of the so-called drug holiday more long-term studies need to be conducted. The reduction in risk may be greatly dependent on the length of the previous bisphosphonate treatment.

Dentoalveolar procedure must be carried out under the prophylactic effect of antibiotics and closed up with a plastic flap and wound suture. In low-risk patients (taking bisphosphonates orally for less than three years with no other risk factors in their medical history), the dentoalveolar procedure can be carried out by their treating doctor. The procedure in medium (oral bisphosphonates for more than three years with other risk factors) or high-risk (cancer patients on i.v. bisphosphonates, denosumab 120 mg/month, combination of high-risk medication, chemotherapy, steroid treatment, etc.) patients has to be carried out by an experienced maxillofacial or dentoalveolar surgeon.

Currently, patients are also prescribed human monoclonal antibodies IgG2 denosumab for treatment of osteological diseases (Prolia, Xgeva), which are similar to bisphosphonates in their antiresorptive effects that are comparable to that of zoledronate, and which might even be stronger than that, according to

some studies [21]. It acts as an immunomodulator because it inhibits osteoclast-mediated bone resorption from extracellular environment. Patients should have it administered once a month subcutaneously and its effect will be felt once it binds to the surface osteoclast receptors. It will not bind to bone tissue and therefore has a relatively short biological half-life and its effect is reversible. The effects of denosumab will disappear one to two months after interrupting the treatment (average half-life after interrupting the treatment is around 28 days). Unlike bisphosphonates, suspending denosumab antiresorptive therapy for one or two months is prognostically justifiable.

### **APPROACH IN PATIENTS WITH DEVELOPED CHEMONECROSIS**

When an exposed alveolar bone, chronic defects, or fistulas on mucous lining are found in a patient on high-risk drugs with positive medical history, they should be sent to a specialist. Treatment and care for patients with pharmaceutical osteonecrosis of the jaw belongs to the competence of maxillofacial surgeons.

### **DISCUSSION**

Osteonecrosis of the jaw is a long-term adverse effect of bisphosphonates. There is currently no evidence in existence which proves that suspension of bisphosphonates treatment reduces the risk of developing the disease. One must admit that the risk is low, with the exception of cancer patients who are administered high doses of intravenous bisphosphonates at more frequent intervals.

The doctor may decide to interrupt the oral bisphosphonates treatment three months before and resume it three months after a dental surgery in order to reduce the risk of developing BON. Suspending intravenously administered bisphosphonates does not provide any short-term benefits.

Interrupting denosumab treatment has prognostic importance because of different pharmacokinetics and reversible effect. According to some published casuistics, the suspension of antiresorptive treatment by denosumab and anti-angiogenic treatment by bevacizumab in patients with developed chemonecrosis led to spontaneous healing of the defect [21].

The treatment of an already developed bisphosphonate-associated osteonecrosis of the jaw is, however, very problematic and lengthy, leaving the patient with an open bone lesion in the oral cavity, which complicates oral care and food intake. Moreover, it also affects the patient's psychological state [8].

In our opinion, the easiest and the most important way to counter this long-term complication is through prevention – thorough examination of oral cavity, including an x-ray and subsequent restoration *before starting the treatment with a high-risk drug*. In addition, one should remember that these preventive measures are already in place for several years in patients who are to undergo a radiotherapy in the maxillofacial region.

A specialist doctor who prescribes a high-risk medication is obliged to inform the patient about possible risks and adverse effects of the planned treatment. Patients often do not know about the adverse effects and locating these high-risk drugs in their medical history may prove a difficult task. Our experience shows that many patients conceal their usage when questioned about the drugs they regularly take. This can be both due to long intervals between administration of such medications (one month, six months, a year), when some patients consider them to be “not so important,” or due to the inability to remember the names of medications they do not use every day.

## CONCLUSION

Osteoporosis is one of the many lifestyle diseases and therefore it can be assumed that the number of patients with the diagnosis will increase in the future and that we will encounter high-risk medication treatment in clinical practice more often. Patients with osteoporosis can be treated with oral or intravenous bisphosphonates and therefore, special attention should be paid to patients in the risk group.

Antiresorptive and anti-angiogenic pharmacotherapy significantly improves the quality of life of such patients. In addition to the benefits, it also has its side effects.

Osteonecrosis of the jaw is the most commonly discussed adverse effect of bisphosphonates therapy in popular and scientific literature.

Each patient should be sent to undergo a dental examination prior to initiation of their bisphosphonates therapy. The patient must be thoroughly examined and rehabilitated by a dentist, including an x-ray and a panoramic radiograph imaging. To reduce the risk of BON development, it is necessary to conduct all invasive procedures, extractions in particular, before the start of the



bisphosphonate treatment. Extractions must be indicated radically, so that the retained teeth would have a good prognosis for several years into the future. Because patients often do not pay enough attention to oral care, it is necessary to educate them about the importance of the subject. Patients need to learn to appear for general medical examinations in time to prevent unavoidable teeth extractions.

Based on the positive experience from around the world, it can be said that observing these precautions will reduce the risk and eliminate the occurrence of osteonecrosis of the jaw in patients who need to take bisphosphonates.

## REFERENCES

- [1] Adam, Z. et al. *Kostná nádorová choroba* [Bone cancer disease]. Prague: Grada Publishing, 2005, ISBN: 80-247-1357-8.
- [2] Allen, M. R., Burr, D. B. The Pathogenesis of Bisfosfonate-Related Osteonecrosis of the Jaw: So many Hypotheses, So Few Data. *J. Oral Maxillofac. Surg.*, 2009, 67(1), pp. 61-70.
- [3] American Association of Oral and Maxillofacial Surgeons Position paper on Bisphosphonate – Related Osteonecrosis of the Jaws. *J. Oral Maxillofac. Surg.*, 2007, 65, pp. 369-376.
- [4] Bisdas, S. et al. Bisphosphonate-induced osteonecrosis of the jaws: CT and MRI spectrum of finding in 32 patients. *Clinical Radiology*. ISSN 0009-9260, 2008, 63(1), pp. 71-77.
- [5] Diel, I. J. et al. Pathophysiology, risk factors and management of bisphosphonate-associated osteonecrosis of the jaw: Is there a diverse relationship of amino- and non-aminobisphosphonates? *Crit. Rev. Oncol. Hematol.*, 2007, 63, pp. 198-207.
- [6] Hagesava, T. et al. The observational study of delayed wound healing after tooth extraction in patients receiving oral bisphosphonate therapy. *Journal of Cranio-Maxillo-Facial Surgery*, 2013, 41, pp. 558-563.
- [7] Hellstein, J., Marek, L. Osteochemonecrosis (Bis-Phossy Jaw): Is This Phossy Jaw of the 21<sup>st</sup> Century? *J. Oral Maxillofac. Surg.* ISSN 0278-2391, 2005, 63(5), pp. 682-689.
- [8] Hodan, R., Mendreková, M., Gruna, J., Cvek, J. Prevence osteonekrózy čelistí před onkologickou léčbou [Prevention of osteonecrosis of the jaw before cancer therapy]. *Onkologie*, 2009, 3(1), pp. 62-65.

- 
- [9] Kennel, K.A., Drake, M.T. Adverse effects of bisphosphonates: Implications for osteoporosis management. *Mayo Clin. Proc.* 2009 July, 84, 7, pp. 632-638.
- [10] Kizek, P., Jenča, A., Havierová, Z. Aktinomykóza I. časť: etiológia a klinický obraz [Actinomycosis Part I: etiology and clinical picture]. *Stomatológ.* ISSN 1335-0005, 2008, 18(3), pp. 4-5.
- [11] Kizek, P., Jenča, A., Havierová, Z. Aktinomykóza II. časť: diagnostika [Actinomycosis Part II: Diagnosis]. *Stomatológ.* ISSN 1335-0005, 2009, 19(2), pp. 12-15.
- [12] Marx, R. E. Pamidronate (Aredia) and Zolendronate (Zometa) induced avascular necrosis of the jaws: A growing epidemic. *J. Oral Maxillofac. Surg.*, 2003, 61, pp. 1115.
- [13] McMahon, R. et al. Osteonecrosis: A multifactorial etiology. *J. Oral Maxillofac. Surg.* ISSN 0278-2391, 2004, 62(7), pp. 904-905.
- [14] Novotný, J. Úloha bisfosfonátu v liečbe pacientu s nádorovými nemocmi (The role of bisphosphonates in the treatment of patients with oncological diseases) [online]. Available online: <<http://www.lpr.cz/index.php/cs/uloha-bisfosfonatu>>.
- [15] Peřina, V. et al. Osteonekróza čelisti při léčbě bisfosfonáty [Osteonecrosis of the jaws in bisphosphonate therapy]. *LKS.* ISSN 1210-3381, 2008, 18(5), pp.140-143.
- [16] Ruggiero, S. L., Dodson, T. B., Assael, L. A., Landesberg, B., Marx, R. E., MEHrota, b. American Association of Oral and Maxillofacial Surgeons Position Paper on Bisphosphonate – Related Osteonecrosis of the Jaws-2009 Update. *J. Oral Maxillofac. Surg.*, 2009, 67(1), pp. 2-12.
- [17] Ruggiero, S. L., Fantasia, J., Carison, E. Bisphosphonate-related osteonecrosis of the jaw: background and guidelines for diagnosis, staging and management. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.*, 2006, 102, pp. 433-441.
- [18] Ruggiero, S. L., Mehrota, B., Rosenberg, T. J., Engroff, S. Osteonecrosis of the jaws associated with the use of bisphosphonates: a review of 63 cases. *J. Oral Maxillofac. Surg.*, 2004, 62, pp. 527-534.
- [19] Špánik, S. Odporúčania Slovenskej onkologickej spoločnosti SLS pre použitie bisfosfonátov v liečbe solídnych nádorov [Recommendations Slovak Oncological Society SLS for the use of bisphosphonates in the treatment of solid tumors]. *Onkológia*, 2009, 4 (4), pp. 249-251.
- [20] Štecová, N. Osteonekróza čeľustných kostí pri liečbe bisfosfonátmi [BON] (Osteonecrosis of the jaw bone in bisphosphonate therapy

[BON])[online]. Available online:

<<http://www.myelom.sk/index.php/kp/bisfosfonaty>>.

[21] <http://www.mkg-update.com/handbuch>.

Affidavit author: The authors declare that the work developed independently using the said literature.

*Chapter 9*

## **THE OXIDATIVE DAMAGE PATHWAYS OF GLUTAMINE IN SOME CLINICAL DISEASES**

***David Calderón Guzmán<sup>1</sup>, Hugo Juárez Olguín<sup>2,3,\*</sup>,  
Ernestina Hernández García<sup>2</sup>, Mónica Punzo Soto<sup>2,3</sup>  
and Gerardo Barragán Mejía<sup>1</sup>***

<sup>1</sup>Laboratorio de Neuroquímica, Instituto Nacional de Pediatría (INP),  
Mexico City, Mexico

<sup>2</sup>Laboratorio de Farmacología, INP, Mexico City, Mexico

<sup>3</sup>Departamento de Farmacología, Facultad de Medicina,  
Universidad Nacional Autónoma de Mexico, Mexico City, Mexico

### **ABSTRACT**

Glutamine is an on-essential amino acid consumed for protein synthesis. It is a substrate for the synthesis of glutathione, the most abundant intracellular thiol and antioxidant. It plays an important role in protecting cells from oxidative stress or apoptosis induced by different diseases. In the present review, we investigated the possible mechanisms of the protective effect of glutamine on animal models and its use in the clinic.

---

\*Corresponding Author: juarezol@yahoo.com.

## INTRODUCTION

Glutamine (Gln) is a conditionally essential aminoacid (Figure1). In the state of metabolic stress, the endogenous biosynthesis of this biomolecule may be insufficient for tissue needs. During chemotherapy and radiotherapy, the administration of Gln is used to restore decreased levels of glutathione[1].

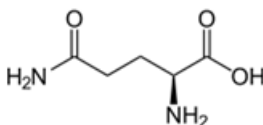


Figure 1. Glutamine.

Its metabolites [alanine (Ala), citrulline (Cit), proline (Pro) and their combination (Ala 10 Pro 4 Cit 1)] are very effective in suppressing the generation of reactive oxygen species (ROS); the release of cytochrome c; and the activation of caspase-3, caspase-8 and caspase-9. Moreover, the multiple physiological functions of these metabolites include the prevention of Ca (2+) influx, activation of calpain, PS exposure and DNA fragmentation as well as the degradation of cytoskeleton, oxidation of membrane and hemoglobin (Hb) and increased activity of anti-hydroxyl radical (AHR) in •OH-induced carp erythrocytes [2]. The accumulation of ROS, which leads to oxidative damage and cell death, plays an important role in a number of neurodegenerative disorders. FOXO3a, the main isoform of FOXO transcription factors, mediates the cellular response to oxidative stress by regulating the expression of genes involved in DNA repair and glutamine metabolism, including glutamine synthase (GS) [3].

Indeed, the inhibition of oxidative stress and apoptosis by Gln in mammals is partly dependent on its metabolites as suggested by the recent studies of Robinson et al, [4]. These authors demonstrated that the inhibition of the activity of glutamine synthase (GS) in lead poisoning is a consequence of protraction in H<sub>2</sub>O<sub>2</sub> clearance, which buttresses the glutathione pathway as the primary therapeutic target in this poisoning. Meiser et al, [5], suggest that the oncogene DJ-1 plays a vital role in cellular antioxidant responses and that their deficient neuronal cells exhibit decreased glutamine influx and reduced serine biosynthesis. Indeed, glutamine withdrawal induces upregulation of phosphoglycerate dehydrogenase (PHGDH) and phosphoserine aminotransferase (PSAT), two enzymes of the serine pathway, where serine is

a key pro-survival actor that needs to be handled to sensitize leukemia cells to glutamine-targeting modalities [6].

## **SUPPLEMENT OF GLUTAMINE**

There is evidence that glutamine is a pharmaco-nutrient and its supplementation in parenteral nutrition improves clinical outcomes in critically ill children or adults [7]. It has been suggested that glutamine supplementation may also benefit preterm infants. Total and reduced glutathione contents in the liver and intestine, and superoxide dismutase activity in the intestine were enhanced by dietary glutamine [8].

A recent study identified 12 randomized controlled trials in which 2877 preterm infants participated. Six of the trials assessed enteral glutamine supplementation and the other six assessed parenteral glutamine supplementation [9]. Although the available trial data do not provide evidence that glutamine supplementation confers important benefits for preterm infants. Glutamine and  $\omega$  3 polyunsaturated fatty acids (PUFAs) are putatively cardio protectors in times of stress of different origins and / or have potential chemo sensitizing effects during cancer chemotherapies [10]. Glutamine prevents in an important way doxorubicin – related deterioration of cardiac function and diminished cardiac lipid peroxidation.

Other results suggest that the protective effect of glutamine on cisplatin is specific for proximal tubular cells and the initial effects may be related to attenuation of cisplatin up take [11].

## **CANCER AND ITS EPIDEMIOLOGY AND TREATMENT**

Oncologic sicknesses in Mexico are the second cause of death in the general population. Deaths by oncologic sicknesses increased 68% from 1953 to 1998. Data from the histopathological register of malignant neoplastic, 1998, showed that in the 90's, an increase in malignant tumor in Mexican adults of 30 years old was registered. 63.5% of the cases were in females and 35.3% in male. Official statistics reported a survival rate of 80% of patients diagnosed and treated of malignant tumors [12]. Despite this report, cancer is presently the principal cause of death in children under 15 years old [13].

**Table 1. Types of cancer diagnoses in pediatric patients**

	Young men		Young women	
	Diagnostics	Cases	Diagnostics	Cases
1	Severally anemia aplastic	1	Metastatic Coriocarcinoma	1
2	Cervical adenopatie	1	Coriocarcinoma ovary	1
3	Colon adenocarcinoma	1	Estesineuroblastoma	1
4	Cadiopatie on genite	2	Ganglioneuroblastoma	1
5	Craneofaringeoma	1	Hepatoesplenomegalia	1
6	Ganglioneuroblastoma suprarenal	1	Hepatoblastoma	2
7	Germinoma	2	Hemangioma of tongue	1
8	Granuloma eosinophilic	1	Hepatoblastoma	1
9	Glioblastoma	1	Histiocytosis	1
10	Hepatoblastoma	3	Acute Lymphoblastic Leukemia	30
11	Histiocytosis	3	Leukemia Granulocytic Acute	1
12	Langerhan's cell Histiocytosis	3	Lymphoblastic Lymphoma	1
13	Leukemia acute	2	Linphagiomalipoblastoma	1
14	Acute Lymphoblastic Leukemia	30	Burkitt's Lymphoma	3
15	Leukemia Acute megakaryocytic	1	Hodgkin's Lymphoma	5
16	Lyphadenopathy submaxilar	1	Diffuse Colon Lymphoma	1
17	Burkitt's Lymphoma	2	Lymphoblastic Lymphoma of bone	1
18	Hodgkin's Lymphoma	9	Medulloblastoma	2
19	Diffuse large B cell Lymphoma	1	Neuroblastoma	4
20	Lymphoma	1	Right Retro-orbital Neuroblastoma	1
21	Lymphoblastic Lymphoma	1	Right femur Osteosarcoma	1
22	Arteriovenous malformation	2	Osteosarcoma linfanoplatia	1
23	Medulloblastoma	4	Osteosarcoma maxilla	1
24	Classic Medulloblastoma	1	Osteoblastic Osteosarcoma	5
25	Neuroblastoma	6	Osteosarcoma of knee	1
26	Osteoblastic Osteoblastoma	1	Osteosarcoma	5
27	Androblastic Osteosarcoma	1	Aneurysmal cyst bone	1
28	Anaplastic Osteosarcoma	1	Rabdomiosarcoma	5

	Young men		Young women	
	Diagnostics	Cases	Diagnostics	Cases
29	Osteoblastic Osteosarcoma	5	Rhabdomyosarcoma alveolar	1
30	Osteosarcoma	6	Rabdomiosarcoma embryo	2
31	Astrocytoma	1	Retinoblastoma	8
32	Pancreatoblastoma	1	Ewing's Sarcoma	12
33	Panhipopituitarismo	1	Pseudocyst cerebral	1
34	Pinealoblastoma	1	Teratoma immature	1
	Young men		Young women	
35	Poliploidia	1	Cystic Teratoma	1
36	Cyst of iliac bone	1	Tumor astropolitik	1
37	Rhabdomyosarcoma	4	Right tumor central parietal	1
38	Alveolar Rhabdomyosarcoma	2	Tumor Endodermic sinus	1
39	Bilateral Retinoblastoma	3	Neuroblastic Tumor of ganglion	1
40	Retinoblastoma	7	Germinal Ovary Tumor	1
41	Orbit Retinoblastoma	1	Tumor mixed germinal	3
42	Ewing's Sarcoma	2	Frontoparietal Tumor	1
43	Axial Ewing's Sarcoma	1	Neuroectodermic Tumor	1
44	Sinovial Sarcoma	1	Tumor of ovary	1
45	Granulocytic Sarcoma	1	Mixedovarian germinal tumor	1
46	Myeloid Sarcoma	1	Polycystic tumor	2
47	Hemophgocytic Syndrome	1	Wilms Tumor	8
48	Schualumamaligne	1	Submandibular Tumor	1
49	Tumor Endodermic sinus	1	Total	131
50	Tumor Endodermic sinus Testicular	1		
51	Mediastinal Tumor	1		
52	Wilms Tumor	4		
53	Fibrous Tumor	2		
54	Germinal Tumor	1		
55	Neuroectodermal Mesenteric Tumor	1		
56	Bone Tumor	1		
57	Left Testicular Tumor	1		
58	Right Testicular Tumor	1		
59	Neuroectodermic Tumor	1		
60	Retro-orbital Tumor	1		
	Total	142		



**Table 2. Amount of chemotherapy pediatric patients administered from January 2005 until December 2007**

		Young men				Young Women			
		2005	2006	2007	Total	2005	2006	2007	Total
1	Folinic acid	44	16	15	75	51	3	2	56
2	Actinomycin	12	86	16	114	24	107	17	148
3	Adriablastine	36	68	75	179	48	79	76	203
4	Amifostin		8	11	19	14	1	9	24
5	Bleomicin	3		11	14		2	3	5
6	Carboplatin	26	50	13	89	18	14	12	44
7	Cetuximab		1		1				
8	Ciclofosfamide	47	166	99	312	77	325	81	483
9	Cisplatin	25	15	49	89	80	28	24	132
10	Arabinoside-C	53	128	96	277	44	180	65	289
11	Dacarbazine	3		11	14		2	2	4
12	Daunorubicin	7	18	19	44	2	9	1	12
13	Etoposide	96	277	204	577	144	314	150	608
14	Ifosfamide	56	129	51	236	117	90	41	248
15	L-Asparaginase	30	78	101	209	34	161	44	239
16	Mannitol	11	5	2	18	38	3	7	48
17	Mesna	64	21	28	113	170	79	26	275
18	Metotrexate	96	210	269	575	73	331	144	548
19	Mercaptopurina			9	9				
20	Oxaliplatin			3	3			1	1
21	Topotecan		1		1	2	25		27
22	Vinblastin	7	116	33	156	1	2	8	11
23	Vincristina	96	213	174	483	79	348	131	558
24	Teniposide					5			5
	Total	712	1606	1289	3607	1021	2103	844	3968

Calderon et al, compared the frequency of different cancer types (Table 1), and chemotherapies prescribed (Table 2) in a pediatric hospital, Mexico City, from January 2005 to December 2007. Table 1 and 2 shows a revision and classification of this information.

Oncological drugs are used either combined or independently in the treatment of different kinds of cancer and oncology diseases with different results. In fact, the prognosis of a patient could change during treatment [14]. The oncological drugs could induce short and long-term adverse effects on organs and tissues that could range from mild to serious damages. In gonads

particularly, these drugs produces alterations in the germinal stem cells with serious and negative consequences on fertility [15].

**Table 3. Studies supporting that Glutamine supplementation ameliorates tissues injury induced by metabolism or external agents on different animal models**

Up date studies	Tissue or animal models	Ref.
Dietary Glutamine (Gln) can improve the intestinal function of the sea cucumbers by increasing the activities of trypsin, lipase and catalase activity.	Sea cucumber <i>Apostichopus japonicus</i>	[17]
Gln's protective effect was significantly weakened by (transforming growth factor- $\beta$ 1) TGF- $\beta$ 1.	Cardiomyocytes	[18]
Diet supplemented with Ala -Gln did not affect the levels of serum glucose, cortisol and catecholamine	Fish	[19]
Pharmacological inhibition of PHD2 recapitulated the adaptation sin glutamine and glycogen metabolism and, consequently, the beneficial effects on cell survival	Periosteal cells	[20]
The combination of apigen in with inhibitors of glutamine metabolism may provide a promising therapeutic strategy for cancer treatment.	Lung cancer cells	[21]
Diets supplemented with 0,0.5,1 and 2% glutamine for 6 weeks, presented higher glutathione peroxidase and glutathione reductase activities and oxidized glutathione content, which seems to reveal a higher glutathione dependency of the intestinal antioxidant response	Intestine	[22]
Gln supplementation dose-dependently elevated reduced-protein thiols in colon with out affecting the level of oxidized- protein thiols	Mice	[23]
Gln supplementation may favorably affect vascular endothelial function in older adults	Human	[24]
Glutamine synthetase providing an alternative perspective for therapeutic intervention in stroke.	Brain	[25]
Gln, by enhancing mitochondrial function and stimulating m TORC1, increases cardiac progenitor cells (CPC) proliferation, and that interventions to increase Gln up take or oxidation may improve CPC therapy.	Cardiac progenitor cells	[26]
Lead intoxication decreases the expression and activity of glutamine synthetase (GS) and supports the glutathione pathway as a primary therapeutic target.	Astrocytes	[27]
Glutamine deprivation induces ROS production, NF- $\kappa$ B activation, and IL-8expression as well as a reduction in GSH	Fibroblasts	[28]

Tumorigenic cells have high proliferative potentials and require higher amount of glutamine and glucose. Visagie et al.,[16], suggest that a short period of glutamine and glucose starvation resulted in decreased cell density, rounded cells and induces apoptosis through the generation of reactive oxygen species and mitochondrial dysfunction, via decreased oxidative stress and inactivation of the intrinsic apoptotic pathway. In this regard, glutamine treatment should be considered as the best option to treat the side effects produced by oncological drugs and abate the proliferative potentials of cancerous cells. Moreover, glutamine supplementation ameliorates tissues injury induced by metabolism or external agents in different studies as shown in Table 3.

## **GLUTAMINE AND BRAIN INJURY**

In the post operative phase, the prognosis of multiple trauma patients with severe brain injuries as well as of patients with extensive head and neck surgery mainly depends on protein metabolism and the prevention of septic complications. Immune- stimulation of patients in the post operative phase is expected to improve their immunological and overall health. Indeed, the authors found that patients who received glutamine-supplemented diets showed total lymphocyte counts and had faster normalization of the percentage of activated CD4+DR+ T helper lymphocytes, in-vitro lymphocyte response to mitogens, as well as IL-2 plasma levels [29]. Enterocytes from very old rats continuously metabolize glutamine into citrulline. This concept was applied in the diagnosis of intestinal atrophy dependent on old age using citrulline as a noninvasive marker [30].

Glutamine is a key substrate for the esplanchnic bed in the whole body. It is a nutrient of particular interest for gastrointestinal tract. Patients with gastrointestinal (GI) cancer are exposed to cachexia, which is highly correlated with chemotherapy-induced side effects. Research suggests that specific immune nutrients could prevent such toxicities, where the efficacy of glutamine and transforming growth factor- $\beta$ 2(TGF- $\beta$ 2) in the prevention of grade 3-4 non-hematological toxicities induced by chemotherapy in patients with GI cancer has been demonstrated [31]. Probably Gln regulates the tight junctions (TJ) integrity through calcium/ calmodulin-dependent kinase 2 (CaMKK2)-AMP-activated protein kinase (AMPK) signaling which, in turn, contributes to improved intestinal mucosal barrier function and altering their intracellular localization in intestinal porcine epithelial cells [32]. Glutamine is the precursor of glutathione, which is an antioxidant and has been demonstrated to improve

out come after several critical illnesses [33]. Its supplementation in critically ill children contributed to maintain high heat shock protein 70 (HSP- 70) levels for longer time [34]. Gln is an important metabolite for the growth of cancer cells. It is the main substrate for DNA and fatty acid synthesis, and reduces the oxidative stress by stimulating the synthesis of glutathione. It stops the process of cancer cachexia, and nourishes the immunological system and the intestine epithelium. The metabolism suggests that the separation of Gln and carbohydrates (Figure2) in the diet can minimize simultaneous supply of ATP from glucose and NADPH<sub>2</sub> from glutamine to cancer cells [1].

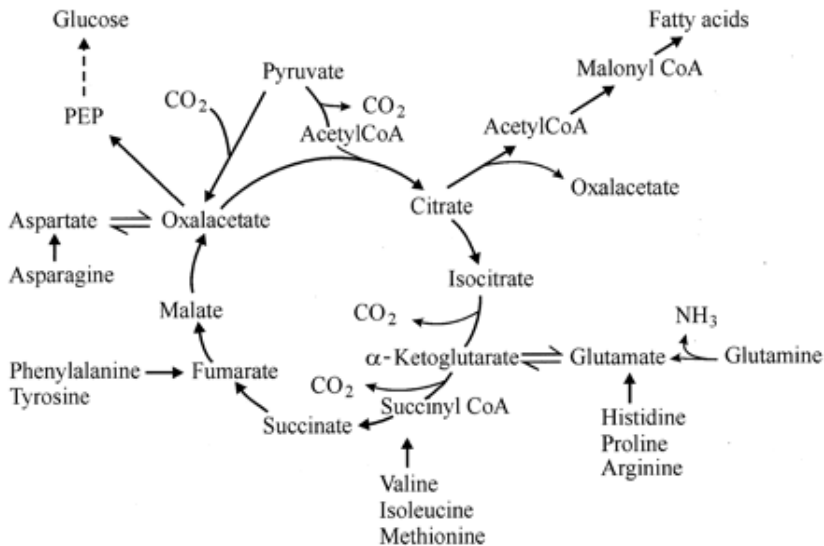


Figure 2. Metabolic separation of carbohydrates and glutamine.

## GLUTAMINE AS PROTECTOR

Indeed, Gln intake restores decreased levels of glutathione in the case of chemotherapy and radiotherapy. Thus, it facilitates the regeneration processes of the intestine epithelium and immunological system. It regulates the expression of inflammatory cytokines such as interleukin 6 and 10, and maintains the balance of the mitochondria in a cytoprotective manner [35]. Preliminary results indicate that glutathione changes the level of ROS in cultured isolated cells and may reduce cancer development [36]. Glutathione

(GSH), glutathione S-transferases (GSTs), and the multi drug resistance-associated protein 1 (MRP1) have been independently studied for their contributions to drug resistance. O'Brien et al, [37], assembled these proteins together, because of their ability to act as a concerted and coordinated pathway, and to increase their expression through single cDNA transfection of GST pi, gamma-glutamyl cysteine synthetase (gamma-GCS) (regulatory plus catalytic subunits), or MRP1 enhanced resistance to a number of anticancer drugs. When all three were transfected, significantly higher levels of resistance were found for doxorubicin and etoposide. Huang et al, [38], suggest that GSTP1 contributes to doxorubicin and cisplatin resistance in osteosarcoma, which may be mediated, in part, by the activation of extracellular signal-regulated kinase 1/2. Targeting of GSTP1 combined with chemotherapy may have synergistic therapeutic effects on osteosarcoma. Excess glutamate at synapses, released in conditions such as traumatic brain injury, can prevent the uptake of cysteine, a necessary building-block of glutathione and glutamine (Figure3).

The protective role of glutathione against cell damage is a well-known fact. GSH plays a critical role in cellular defense against electrophiles, oxidative stress and nitrosating species. Administration of L-cysteine precursors and other strategies allow GSH levels to be maintained under conditions that would otherwise result in GSH depletion and cytotoxicity (Figure 4). Conversely, inhibitors of gamma-GCS have been used to deplete GSH as a strategy for increasing the sensitivity of tumors and parasites to certain therapeutic interventions [39].

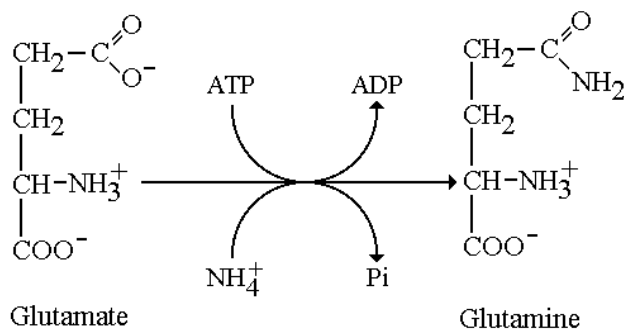


Figure 3. Glutamate as precursor of glutamine.

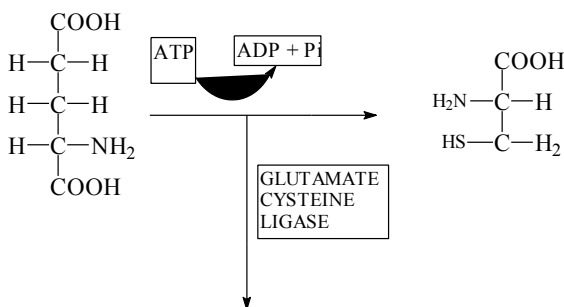


Figure 4. Glutamate, Cysteine.

## NUTRITIONAL STATE

However, once a cancer is developed, by conferring resistance to a number of chemotherapeutic drugs, elevated levels of glutathione in tumor cells are able to protect cancerous cells in bone marrow, breast, colon, larynx, and lung cancers [40].

When patients are admitted in the Intensive Care Unit in the hospital due to health complications as consequence of any disease, it is common that the clinical conditions of nutrition be found in deficiency in many cases even when the minimum daily requirements had been met [41]. Deficient ingestion of nutrients affects all body cells in varying degrees, with repercussions in different organs and systems, thereby modifying the capacity of the organism to respond to aggressors of diverse nature.

Nutritional oxidative stress describes an imbalance between the pro-oxidant load and the antioxidant defense. Such imbalance is mainly because of excess oxidative load or of inadequate supply of nutrients to the organism [42]. Thus, when malnutrition is present, it is expected that the imbalance between production of free radicals and their safe disposal through the activation of antioxidant enzymes or antioxidant nutrients promotes increased oxidative stress [43]. Nutritional recovery is a complex process, which is not free of complications, because not only the amount of nutrients but also the type and form of administration, are determinants [44]. Experimental studies have shown that the selective administration of nutrients, and the addition of some supplements, tends to show positive results in animals that have been subjected to malnutrition [45].

With the recognition that nutritional recovery is not just a feed-related procedure, the clinical management of malnutrition, has incorporated focused strategies to deal with specific aspects of malnutrition that could affect the rehabilitation of the whole organism. The development to clinical strategies targeted to the reduction of the risk of suffering additional damage by oxidative stress during nutritional recovery has become very important issue.

## GLUTAMINE, THE EXPECTANCE

Studies carried out in recent years, strongly suggest that a therapy focused on restoring the antioxidant capacity by applying glutamine equivalents in the form of glutathione is beneficial for biochemical and clinical recovery. Indeed, it is of great importance to consider the incorporation of antioxidants in the diet during nutritional recovery in the clinical management of chemotherapies, malnutrition and any disease.

## REFERENCES

- [1] Michalak, K. P., Maćkowska-Kędziora, A., Sobolewski, B., Woźniak, P.: Key roles of glutamine pathways in reprogramming the cancer metabolism. *Oxid. Med. Cell. Longev.* 2015, 964321.
- [2] Li, H., Jiang, W., Liu, Y., Jiang, J., Zhang, Y., Wu, P., Zhao, J., Duan, X., Zhou, X., Feng, L.: The metabolites of glutamine prevent hydroxyl radical-induced apoptosis through inhibiting mitochondria and calcium ion involved pathways in fish erythrocytes. *Free Radic. Biol. Med.* 2016, 92:126-40.
- [3] Fluteau, A., Ince, P. G., Minett, T., Matthews, F. E., Brayne, C., Garwood, C. J., Ratcliffe, L. E., Morgan, S., Heath, P. R., Shaw, P. J., Wharton, S. B., Simpson, J. E.: MRC cognitive function ageing. Neuropathology Study Group. The nuclear retention of transcription factor FOXO3a correlates with a DNA damage response and increased glutamine synthetase expression by astrocytes suggesting a neuroprotective role in the ageing brain. *Neurosci. Lett.* 2015, 609:11-17.
- [4] Robinson, S. R., Lee, A., Bishop, G. M., Czerwinska, H., Dringen, R.: Inhibition of astrocytic glutamine synthetase by lead is associated with as

- lowed clearance of hydrogen peroxide by the glutathione system. *Front. Integr. Neurosci.* 2015, 9:61.
- [5] Meiser, J., Delcambre, S., Wegner, A., Jäger, C., Ghelfi, J., d'Herouel, A. F., Dong, X., Weindl, D., Stautner, C., Nonnenmacher, Y., Michelucci, A., Popp, O., Giesert, F., Schildknecht, S., Krämer, L., Schneider, J. G., Woitalla, D., Wurst, W., Skupin, A., Weisenhorn, D. M., Krüger, R., Leist, M., Hiller, K.: Loss of DJ-1 impairs antioxidant response by altered glutamine and serine metabolism. *Neurobiol. Dis.* 2016, 89:112-125.
- [6] Polet, F., Corbet, C., Pinto, A., Rubio, L. I., Martherus, R., Bol, V., Drozak, X., Grégoire, V., Riant, O., Feron, O.: Reducing the serine availability complements the inhibition of the glutamine metabolism to block leukemia cell growth. *Oncotarget*, 2016, 7:1765-1776.
- [7] Leguina-Ruzzi, A.: A commentary on the 2015 Canadian Clinical Practice Guidelines. In: glutamine supplementation to parenteral nutrition. *Crit. Care*, 2016, 20:7.
- [8] Coutinho, F., Castro, C., Rufino-Palomares, E., Ordóñez-Grande, B., Gallardo, M. A., Oliva-Tels, A.: Dietary glutamine supplementation effects on amino acid metabolism, intestinal nutrient absorption capacity and antioxidant response of gilthead sea bream (*Sparus aurata*) juveniles. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 2016, 191:9-17.
- [9] Moe-Byrne, T., Brown, J. V., Mc Guire, W.: Glutamine supplementation to prevent morbidity and mortality in preterm infants. *Cochrane Data base Syst. Rev.* 2016, 12:1.
- [10] Xue, H., Ren, W., Denking, M., Schlotzer, E., Wischmeyer, P. E.: Nutrition modulation of cardiotoxicity and anticancer efficacy related to doxorubicin chemotherapy by glutamine and  $\omega$ -3 polyunsaturated fatty acids. *J. Parenter. Enteral. Nutr.* 2016, 40:52-66.
- [11] Kim, H. J., Park, D. J., Kim, J. H., Jeong, E. Y., Jung, M. H., Kim, T. H., Yang, J. I., Lee, G. W., Chung, H. J., Chang, S. H.: Glutamine protects against cisplatin-induced nephrotoxicity by decreasing cisplatin accumulation. *J. Pharmacol. Sci.* 2015, 127: 117-26.
- [12] Mejia-Aranguré, J. M., Ortega-Alvarez, M. C., Fajardo-Gutiérrez, A.: Epidemiology of acute leukemia in children. Part 1. *Rev. Med. IMSS.* 2005, 43:323-333.
- [13] Ross, J. A., Severson, R. K., Pollock, B. H.: Childhood cancer in the Unites States. *Cancer* 1996, 77:201-207.
- [14] Prostate cancer: Guide for patients treatment. NCCN and American Cancer Society. USA. 2005, 64.



- [15] Gerson, R.: Fertility and cancer. *Rev. Med. Hosp. Gral. México*. 2000, 63: 30-40.
- [16] Visagie, M. H., Mqoco, T. V., Liebenberg, L., Mathews, E. H., Mathews, G. E., Joubert, A. M.: Influence of partial and complete glutamine and glucose deprivation of breast and cervical tumorigenic cell lines. *Cell. Biosci.* 2015, 5:37.
- [17] Yu, H., Gao, Q., Dong, S., Lan, Y., Ye, Z., Wen, B.: Regulation of dietary glutamine on the growth, intestinal function, immunity and antioxidant capacity of sea cucumber *Apostichopus japonicus* (Selenka). *Fish Shellfish Immunol.* 2016, 50:56-65.
- [18] Zhang, H., Cui, Y. C., Li, K., Yang, B. Q., Liu, X. P., Zhang, D., Li, H., Wu AL, Tang Y. Glutamine protects cardiomyocytes from hypoxia / reoxygenation injury under high glucose conditions through inhibition of the transforming growth factor -  $\beta$ 1 –Smad3 pathway. *Arch. Biochem. Biophys.* 2016, Mar 3.
- [19] Chen, X. M., Guo, G. L., Sun, L., Yang, Q. S., Wang, G. Q., Q in, G. X.: Effects of Ala-Gln feeding strategies on growth, metabolism, and crowding stress resistance of juvenile *Cyprinus carpio* var. *Fish Shellfish Immunol.* 2016, Mar 2.
- [20] Stegen, S., van Gastel, N., Eelen, G., Ghesquière, B., D'Anna, F., Thienpont, B., Goveia, J., Torrekens, S., Van Looveren, R., Luyten, F. P., Maxwell, P. H., Wielockx, B., Lambrechts, D., Fendt, S. M., Carmeliet, P., Carmeliet, G.: HIF - 1 $\alpha$  promotes glutamine – mediated redox homeostasis and glycogen – dependent bioenergetics to support post implantation bone cell survival. *Cell. Metab.* 2016, 23:265-279.
- [21] Lee, Y. M., Lee, G., Oh, T. I., Kim, B. M., Shim, D. W., Lee, K. H., Kim, Y. J., Lim, B. O., Lim, J. H.: Inhibition of glutamine utilization sensitizes lung cancer cells to a pigenin – induced apoptosis resulting from metabolic and oxidative stress. *Int. J. Oncol.* 2016, 48:399-408.
- [22] Coutinho, F., Castro, C., Rufino-Palomares, E., Ordóñez-Grande, B., Gallardo, M. A., Oliva-Teles, A., Peres, H.: Dietary glutamine supplementation effects on amino acid metabolism, intestinal nutrient absorption capacity and antioxidant response of gilthead sea bream (*Sparus aurata*) juveniles. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 2016, 191:9-17.
- [23] Chaudhry, K. K., Shukla, P. K., Mir, H., Manda, B., Gangwar, R., Yadav, N., Mc Mullen, M., Nagy, L. E., Rao, R.: Glutamine supplementation attenuates ethanol-induced disruption of apical junctional complexes in

- colonic epithelium and ameliorates gut barrier dysfunction and fatty liver in mice. *J. Nutr. Biochem.* 2016, 27:16-26.
- [24] Ellis, A. C., Patterson, M., Dudenbostel, T., Calhoun, D., Gower, B.: Effects of 6-month supplementation with  $\beta$ -hydroxy- $\beta$ -methylbutyrate, glutamine and arginine on vascular endothelial function of older adults. *Eur. J. Clin. Nutr.* 2016, 70:269-73.
- [25] Jeitner, T. M., Battaile, K., Cooper, A. J.: Critical evaluation of the changes in glutamine synthetase activity in models of cerebral stroke. *Neurochem. Res.* 2015, 40:2544-2556.
- [26] Salabei, J. K., Lorkiewicz, P. K., Holden, C. R., Li, Q., Hong, K. U., Bolli, R., Bhatnagar, A., Hill, B. G.: Glutamine regulates cardiac progenitor cell metabolism and proliferation. *Stem. Cells.* 2015, 33:2613-2627.
- [27] Robinson, S. R., Lee, A., Bishop, G. M., Czerwinska, H., Dringen, R.: Inhibition of astrocytic glutamine synthetase by lead is associated with as lowed clearance of hydrogen peroxide by the glutathione system. *Front. Integr. Neurosci.* 2015, 9:61.
- [28] Kim, M.H., Kim, A., Yu, J. H., Lim, J. W., Kim, H.: Glutamine deprivation induces interleukin -8 expression in ataxia telangiectasia fibroblasts. *Inflamm. Res.* 2014, 63:347-56.
- [29] Lorenz, K. J., Schallert, R., Daniel, V.: Immunonutrition – the influence of early post operative glutamine supplementation in enteral/ parenteral nutrition on immune response, wound healing and length of hospital stay in multiple trauma patients and patients after extensive surgery. *GMS Interdiscip. Plast Reconstr. Surg. DGPW.* 2015, 15:4.
- [30] Meynial-Denis, D.: Glutamine metabolism in advanced age. *Nutr. Rev.* 2016, Mar 2.
- [31] Khemissa, F., Mineur, L., Amsellem, C., Assenat, E., Ramdani, M., Bachmann, P., Janiszewski, C., Cristiani, I., Collin, F., Courraud, J., de Forges, H., Dechelotte, P., Senesse, P.: Aphase III study evaluating oral glutamine and transforming growth factor-beta 2 onc hemotherapy – induced toxicity in patients with digestive neoplasm. *Dig. Liver Dis.* 2016, 48:327-332.
- [32] Wang, B., Wu, Z., Ji, Y., Sun, K., Dai, Z., Wu, G.: l-Glutamine enhance stight junction integrity by activating Ca M K kinase2-AMP-activated protein kinase signaling in intestinal porcine epithelial cells. *J. Nutr.* 2016, 146:501-508.
- [33] Aydin, M., Yildiz, A., Ibiloglu, I., Ekinci, A., Ulger, B. V., Yuksel, M., Bilik, M. Z., Ozaydogdu, N., Ekinci, C., Yazgan, U. C.: The protective

- role of glutamine against acute induced toxicity in rats. *Toxicol. Mech. Methods* 2015, 25:296-301.
- [34] Jordan, I., Balaguer, M., Esteban, M. E., Cambra, F. J., Felipe, A., Hernández, L., Alsina, L., Molero, M., Villaronga, M., Esteban, E.: Glutamine effects on heat shock protein 70 and interleukins 6 and 10: Randomized trial of glutamine supplementation versus standard parenteral nutrition in critically ill children. *Clin. Nutr.* 2016, 35:34-40.
  - [35] Safi, S. Z., Batumalaie, K., Mansor, M., Chinna, K., Mohan, S., Karimian, H., Qvist, R., Ashraf, M. A., Yan, G. O.: Glutamine treatment attenuates hyperglycemia – induced mitochondrial stress and apoptosis in umbilical vein endothelial cells. *Clinics (Sao Paulo)* 2015, 70:569-576.
  - [36] Balendiran, G. K., Dabur, R., Fraser, D.: The role of glutathione in cancer. *Cell. Biochem. Funct.* 2004, 22: 343-52.
  - [37] O'Brien, M., Kruh, G. D., Tew, K. D.: The influence of coordinate over expression of glutathione phase II detoxification gene products on drug resistance. *J. Pharmacol. Exp. Ther.* 2000, 294:480-487.
  - [38] Huang, G., Mills, L., Worth, L. L.: Expression of human glutathione S-transferase P1 mediates the chemosensitivity of osteosarcoma cells. *Mol. Cancer Ther.* 2007, 6:1610-9.
  - [39] Griffith, O. W.: Biologic and pharmacologic regulation of mammalian glutathione synthesis. *Free Radic. Biol. Med.* 1999, 27:922-935.
  - [40] Chow, H. H. S., Hakim, I. A., Vining, D. R., Crowell, J. A., Tome, M. E., Ranger-Moore, J., Cordova, C. A., Mikhael, D. M.: Modulation of human glutathione S-transferases by polyphenon E intervention. *Cancer Epidemiol. Biomark. Prevention* 2007, 16:1662-1666.
  - [41] Calderón, G. D., Hernández, G. E., Osnaya, B. N., Trujillo, J. F., Barragán, M. G., Juárez, O. H., Santamaría, D. D., Nuñez, A. E., Carmona, A. L.: Effect of oseltamivir on catecholamines and select oxidative stress markers in the presence of oligoelements in the rat brain. *Arch. Pharm. Res.* 2010, 33:1671-1677.
  - [42] Sies, H., Stahl, W., Sevanian, A.: Nutritional, dietary and post prandial oxidative stress. *J. Nutr.* 2005, 135:969-972.
  - [43] Fechner, A., Böhme, C., Gromer, S., Funk, M., Schirmer, R., Becker, K.: Antioxidant status and nitric oxide in the mal nutrition syndrome kwashiorkor. *Pediatr. Res.* 2001, 49:237-243.
  - [44] Adkins, S. M., Fürst, P.: New concepts on nutritional management of severe malnutrition: the role of protein. *Curr. Opin. Clin. Nutr. Metab. Care* 2000, 3:31-38.

- 
- [45] Ventrucchi, G., Ramos, S. L. G., Roston, M. M. A.: Effects of aleucine-rich diet on body composition during nutritional recovery in rats. *Nutrition* 2004, 20:213-217.



*Chapter 10*

## GLUTAMINE IN SPORT AND EXERCISE

***Hércules Rezende Freitas\****

Laboratory of Neurochemistry, Carlos Chagas Filho Institute  
of Biophysics, Federal University of Rio de Janeiro (UFRJ),  
Rio de Janeiro, Brazil

### ABSTRACT

L-glutamine, a nonessential neutral amino acid, is a key element in several metabolic pathways. Recent investigations have been focused mainly on energy metabolism, cellular proliferation and immune functions of glutamine. Athletes and exercise practitioners regularly supplement this amino acid as a resource to optimize physical performance and prevent immune impairment. Also, studies indicate that acute doses of glutamine, reaching up to 0.65 g/Kg/day (45.5 g for a 70 Kg individual), are well tolerated and do not result in abnormal ammonia levels. This chapter presents relevant features of the glutamine role in immune pathways, energy metabolism and exercise, highlighting the relationships between glutamine supplementation and performance in adult subjects with different levels of activity. In sport, data suggest that glutamine supplementation may increase tolerance to intermittent exercise, lower levels of fatigue, enhance physical and performance measurements,

---

\* Corresponding author: Hércules Rezende Freitas. Federal University of Rio de Janeiro, Center for Health Sciences, Institute of Biophysics Carlos Chagas Filho, Cidade Universitária - 21.941-902 - Rio de Janeiro, RJ – Brazil. Tel: +55(21)9.8612-2194. E-mail: freitashr@biof.ufrj.br/herculesrezendef@hotmail.com.

optimize recovery from muscle damage and prevent suppression of neutrophil function, however, these effects are highly dependent on the characteristics of exercise protocols, experimental subjects and doses provided. Data indicating higher levels of nasal and intestine IgA, prevention of hyperammonemia, protection against lymphocyte apoptosis, increase in exercise-induced plasma IL-6 levels and inhibition of cytokines production (NF- $\kappa$ B pathway) through glutamine supplementation suggest a mechanism of immune communication, and novel research strongly points to the importance of immunological exchange in the modulation of performance. Glutamine, however, may be deleterious to the nervous system under disease conditions, such as in hyperammonemia associated with acute liver failure, where excess glutamine leads to cerebral edema. Therefore, supplementation or pharmacological interventions should be weighted according to one's health status and level of activity.

**Keywords:** glutamine, immune system, physical activity, metabolism

## INTRODUCTION

Eric Newsholme and coworkers have provided, in the late 80's, the first concrete view of L-glutamine (GL) as a key modulator of the immune system (E. Newsholme, Newsholme and Curi, 1987). Initial observations showed that GL is used as intermediate in several metabolic pathways, such as  $\alpha$ -ketoglutarate in tricarboxylic acid cycle, which can be obtained from direct GL metabolism or from glutamate deamination (Curthoys, 2001). Lymphocytes, macrophages, and neutrophils have high rates of glutamine utilization, which is especially important for proliferation of activated cells and production of inflammatory mediators (P. Newsholme et al., 1999; P. Newsholme, Curi, Gordon and Newsholme, 1986).

Knowledge on GL function as an adjuvant of immune responses raised discussion over its potential role in non-infectious stress mechanisms, such as in cancer, degenerative and metabolic diseases (P. Newsholme, 2001). In fact, glutamine has been shown to promote cell resilience and optimize outcome of patients exposed to chemotherapy and radiation treatments (Kuhn, Muscaritoli, Wischmeyer and Stehle, 2010). Recent research, however, have explored GL as a potential marker for physiological responses to physical activity. Further, GL supplementation has been used as an ergogenic resource to promote exercise recovery and optimize performance parameters in competitive sports, reviewed in (H.R. Freitas, da Silva Pereira and da Silva Ramos, 2015).

**Table 1. Characteristics and outcomes of studies applying glutamine supplementation in sport and exercise**

Author	Age	Sex	Protocol	Results
(Favano et al., 2008)	18.4 ± 1.1	Male	3.5 g glutamine peptide plus 50 g of maltodextrin (Ac)	Increase in athlete's distance and duration of tolerance to intermittent exercise, and lowered feelings of fatigue among players when compared with the use of carbohydrates alone.
(Antonio, Sanders, Kalman, Woodgate and Street, 2002)	21.5 ± 0.3	Male	22.95 g <sup>l</sup> glutamine (Ac)	Short-term glutamine ingestion had no effect on muscular strength and weightlifting performance.
(Colker, Swain, Fabrucini, Qiuhi and Kalman, 2000)	32.2 ± 8.0	Male	5 g of glutamine plus 3 g of branched chain amino acids (BCAA) and 40 g of whey protein (Ch)	Supplementing the diet of resistance-trained athletes engaged in a hypertrophy-training regimen with whey protein, glutamine and BCAA, enhanced physical and performance measures.
(Sasaki et al., 2013)	19.4 ± 0.6	Male	3 g of glutamine (Ch)	Glutamine supplementation contributed to earlier recovery from muscle damage and on preventing suppression of neutrophil function, especially in production of ROS, even during a highly intensive training period.
(Krieger, Crowe and Blank, 2004)	29.1 ± 2.8	Male (9) and Female (4)	27.96 g <sup>l</sup> of glutamine (Ch)	Chronic, high-dose glutamine supplementation had no effect on salivary IgA concentration or plasma glutamine concentration in resting athletes. Supplementation resulted in higher nasal IgA during training.
(Cury-Boaventura et al., 2008)	24.9 ± 4.0	Male	175 mg of glutamine dipeptide plus 2.8 g of hydrolyzed whey protein and 50 g of maltodextrin (Ac)	Maltodextrin plus hydrolyzed whey protein enriched with glutamine dipeptide supplementation, previous to exercise, partially prevented apoptosis of human lymphocytes, possibly by a protective effect on mitochondrial function.



**Table 1. (Continued)**

Author	Age	Sex	Protocol	Results
(Khorshidi-Hosseini and Nakhostin-Roohi, 2013) <sup>2</sup>	23.29 ± 3.53 and 22.14 ± 2.85	Male	20.53 g <sup>1</sup> of glutamine peptide plus 30 g of sweetener or 18.5 g <sup>1</sup> of glutamine peptide plus 50 g of maltodextrin and 30 g of sweetener (Ac)	Supplementation of glutamine and maltodextrin combination, 2 hours before exercise is more efficient in prevention of anaerobic power decrease than consumption of carbohydrate or glutamine alone in repeated bouts of Running-based Anaerobic Sprint Test protocol. Thus, supplementation with both carbohydrate and glutamine peptide improved the physical performance of athletes during repeated competitions.
(Hoffman et al., 2012)	21.2 ± 1.6	Female	1 g of glutamine peptide <sup>3</sup> or 2 g of glutamine peptide <sup>3</sup> (Ac)	Rehydration with glutamine peptide <sup>3</sup> appears to maintain basketball skill performance and visual reaction time to a greater extent than water only.
(Hoffman et al., 2010)	20.8 ± 0.6	Male	15.48 g <sup>1</sup> of glutamine peptide <sup>3</sup> or 3.87 g <sup>1</sup> of glutamine peptide <sup>3</sup> (Ac)	Glutamine peptide <sup>3</sup> supplementation increased time to exhaustion during a mild hydration stress through an enhanced fluid and electrolyte uptake. Glutamine peptide supplementation did not have any effect on immune, inflammatory or oxidative stress responses. Results also indicate that glutamine peptide supplement did not influence the pituitary adrenal-testicular axis during exercise and mild hypohydration perturbation.
(Krzywkowski et al., 2001)	38 <sup>4</sup>	Male	17.5 g of glutamine or 68.5 g of protein (6.2 g of protein-bound glutamine) or 17.5 g of maltodextrin (Ac)	Exercise-induced change in salivary IgA level was not affected by glutamine or protein supplements. The study therefore found no support for the glutamine hypothesis, which says that exercise-induced decrease in plasma glutamine is linked to postexercise immune impairment.

Author	Age	Sex	Protocol	Results
(Hiscock et al., 2003)	36.5 <sup>4</sup>	Male	17.5 g of glutamine or 68.5 g of protein (6.2 g of protein-bound glutamine) or 17.5 g of maltodextrin (Ac)	The study demonstrated that exercise-induced increase in plasma IL-6 is further enhanced by glutamine supplementation.
(Bassini-Cameron, Monteiro, Gomes, Werneck-de-Castro and Cameron, 2008) <sup>2</sup>	22.6 ± 0.6	Male	7.08 g <sup>1</sup> of glutamine (Ac-Ch)	Five days of glutamine supplementation prior to exercise partially prevents hyperammonemia observed after both intermittent and continuous exercise.
(Carvalho-Peixoto, Alves and Cameron, 2007)	35.5 ± 9.8	Male	4.4 g <sup>1</sup> (70 mg/Kg) glutamine with or without 1 g/Kg carbohydrate	Glutamine, carbohydrates, or both attenuated by 15% overall ammonia increase throughout a 60-min exercise protocol.
(Pruna et al., 2014)	23.5 ± 3.7	Male	Sports drink plus 600 or 1000 mg/L glutamine peptide <sup>3</sup> (Ac)	Rehydration with a commercial sports drink plus 600-1000 mg/L glutamine peptide <sup>3</sup> , when compared to no hydration, enhanced reaction time in lower and upper body activities following endurance exercise.
(Zuhl et al., 2015)	26 ± 4.4	Male (2) and Female (5)	Sugar-free lemon drink plus ± 40 g (0.9 g/Kg of free-fat mass) glutamine (Ac)	Exercise-induced intestinal permeability and pro-inflammatory cytokine production (NF-κB pathway) were attenuated with previous-to-exercise acute glutamine supplementation.
(Zuhl et al., 2014)	25 ± 4.0	Male (5) and Female (3)	Sugar-free lemon drink plus ± 48 g (0.9 g/Kg of free-fat mass) glutamine (Ch)	Chronic high-dose glutamine supplementation protects intestinal epithelial cell barrier through activation of heat shock pathways (HSF-1 and HSP70) and inhibition of NF-κB inflammatory mechanisms.
(da Silveira et al., 2014)	24 <sup>4</sup>	Male	Sweetened beverage containing 0.03 g/Kg glutamine (Ch)	Chronic glutamine supplementation (three-month protocol) did not improve aerobic/anaerobic capacity, upper and lower limb muscular strength, flexibility and local muscle endurance.

**Table 1. (Continued)**

Author	Age	Sex	Protocol	Results
(Kaldirimci et al., 2015)	20.4 ± 1.6	Male	2 or 4 g/L glutamine solution in water (Ac)	Rehydration with glutamine throughout basketball trials promoted a dose-dependent enhancement of game performance and hydration, optimizing reaction time and shooting skills.
(Ramallo et al., 2013)	19.9 ± 1.0	Male	50 g maltodextrin plus 1440 mg glutamine peptide (Ac)	Glutamine supplementation prior to a resistance training session did not prevent muscle damage in untrained individuals. Also, lower leukocyte counting were observed in the supplemented group 24 h after application of training protocol.
(Ionescu, Vasilescu, Carmoci, Nica and Ionescu, 2014)	21.3 ± 1.3	Female	300 mg/Kg/day glutamine from hydrolyzed wheat protein (Ch)	Chronic (three-month protocol) glutamine supplementation improved aerobic performance and recovery rate of highly trained female gymnasts.

<sup>1</sup> Doses of glutamine, when provided in g/kg of body weight, were calculated by mean weight between individual participants. <sup>2</sup> More than one supplementation protocol was used by the authors. <sup>3</sup> L-alanyl-L-glutamine form. <sup>4</sup> Data do not provide Standard Deviation. Individuals received acute (Ac), chronic (Ch) or either (Ac-Ch) glutamine supplementation. Adapted from Freitas et al., 2015.

High intensity and prolonged exercise is common in professional sport, and the resultant metabolic stress may chronically lower plasma GL levels. Experimental data indicate that GL concentrations suffer an observable decrease 1 h after performing a marathon competition (Castell and Newsholme, 1997). Also, authors report that short-term high intensity exercise promoted significant attenuation of GL levels throughout 10 days of training trial, while a followed six-day recovery period partially restored basal GL concentrations, indicating that GL is an efficient exercise-induced stress marker (Keast, Arstein, Harper, Fry and Morton, 1995; Rowbottom, Keast and Morton, 1996).

Although GL decline has been widely applied as a stress marker (Walsh, Blannin, Robson and Gleeson, 1998), a consensus whether supplementation would help prevent exercise-induced immune suppression has not been reached. Michael Gleeson, in a previous review, indicates that GL supplementation lack necessary evidence for supporting the idea that such resource would prevent immune impairment, and also suggest that carbohydrates, but not GL or antioxidant vitamins, may modulate rises in stress markers, such as cortisol (Gleeson and Bishop, 2000; Halson, Lancaster, Achten, Gleeson and Jeukendrup, 2004). Despite low influence on stress and immune parameters, recent data positively correlates GL supplementation with improvements in sports performance and muscular recovery (H.R. Freitas et al., 2015; Hoffman et al., 2012; Kaldirimci, Sajedi, Sam, Mizrak and Kavurmaci, 2015; Legault, Bagnall and Kimmerly, 2015).

Here, we discuss emerging and well-established roles of GL in immune pathways, energy metabolism and exercise, highlighting the relationships between glutamine supplementation and performance in adult subjects, discussing the possible involvement of GL in the modulation of biomarkers relevant for exercise recovery and long term performance.

## PERFORMANCE IMPROVEMENT

A myriad of works have evaluated the role of GL in sport and exercise, mainly its potential benefits in immune impairment, muscle recovery, hydration, endocrine and metabolic markers, which would be correlated to enhancements in performance and competitive advantage. Table 1 presents summarized information regarding characteristics and outcomes from recent original works. It's relevant to notice the low number of investigations with female participants, only 5 works in 20 explored the effects of GL in female groups, highlighting the

need for original protocols to elucidate whether GL would promote gender-specific outcomes.

Another important feature of most works regarding GL supplementation in physical activity is the absence of elderly individuals. López-Otín et al. (2013) list several hallmarks for the ageing process, including altered intracellular communication, genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and deregulated nutrient sensing (López-Otín, Blasco, Partridge, Serrano and Kroemer, 2013).

All these phenomena have relevant impact on the subject's response to exercise, and GL supplementation may work as a special resource in the fight against organic senescence and prevention of degenerative diseases. Galera et al. (2010) did not observe differences in ammonemia levels of elderly individuals supplemented with 0.5 g/Kg/day GL doses. Although statistically greater, urea nitrogen and creatinine concentrations were not of clinical significance, indicating that GL supplements may be provided to the elderly, since kidney health parameters are previously observed with caution (Galera et al., 2010).

GL supplementation increased growth hormone (GH) in elderly individuals without interfering in mood or memory parameters (Arwert, Deijen and Drent, 2003). Acute physiology and chronic health evaluation (APACHE) and multiple organ dysfunction syndrome (MODS) scores were decreased, and total lymphocyte count and number of HLA-DR-expressing CD14<sup>+</sup> monocytes were increased after providing elderly patients with GL-enriched nutritional support (Cai, Yan, Zhang and Yu, 2008). A report from Blomqvist et al. (1995) indicate that elderly patients, after undergoing hip replacement, have greater levels of plasma amino acids and protein synthesis if supplemented with 2 g/Kg GL, preventing damage from surgical trauma (Blomqvist, Hammarqvist, von der Decken and Wernerman, 1995).

Regarding GL effects on the performance of adult active individuals, collective data frequently suggest a positive role on several exercise parameters. As previously discussed, carbohydrates are strong candidates for inducing attenuation of stress response to physical activity (Gleeson and Bishop, 2000), and GL may act as an adjuvant in promoting endurance gain throughout sports practice, as shown when professional soccer players were provided with 50g maltodextrin plus 3.5 g GL peptide supplementation within a sports beverage, ingested throughout evaluation trials (Favano et al., 2008). A study supplementing higher doses ( $\pm 20$  g) of GL peptide, maltodextrin (50 g) or both showed that either GL or maltodextrin were efficient in attenuating loss of

anaerobic power, and that combining both supplements further increased gains in a sprint performance test (Khorshidi-Hosseini and Nakhostin-Roohi, 2013).

Cury-Boaventura et al. (2008) provided male subjects with 50 g maltodextrin, 175 mg GL peptide and 2.8 g hydrolyzed whey protein previous to an exercise protocol, indicating partial prevention of lymphocyte death (Cury-Boaventura et al., 2008). It must be noticed, however, that carbohydrates may be the main element in preventing stress-induced cell death, not GL. Interestingly, a recent work in which athletes were provided with both maltodextrin (50 g) and GL peptide (1440 mg) previously to an exercise protocol showed lower leukocyte counting in the supplemented group 24 h after completion of training (Ramallo et al., 2013). A third possibility is that hydrolyzed whey protein, provided only in the study from Cury-Boaventura, is responsible for the partial prevention of lymphocyte death, as reviewed in (Gauthier, Pouliot and Saint-Sauveur, 2006).

GL (17.5 g), protein (68.5 g, with 6.2 g of protein-bound glutamine) or maltodextrin (17.5 g) were unable to influence exercise-induced change in salivary IgA levels (Krzywkowski et al., 2001). Using the same supplementation protocol, Hiscock et al. (2003) demonstrated that exercise-induced increase in plasma IL-6 is further enhanced by glutamine supplementation (Hiscock et al., 2003). This result, however, not necessarily reflect a GL action over immune cells. As previously reported, skeletal muscle cells produce and release large amounts of IL-6 throughout exercise (Steensberg et al., 2002), an GL supplementation may be able to stimulate this process.

Ramallo et al. (2013) provided subjects with 50 g maltodextrin plus 1440 mg GL peptide prior to a resistance training session. Supplementation were unable to prevent muscle damage in untrained participants (Ramallo et al., 2013). A chronic GL (3 g/day) treatment, however, were recently shown to promote muscle damage recovery, preventing performance decline after high intensity exercise (Sasaki et al., 2013). As previously highlighted, exercise-induced muscle damage occurs as a result of unaccustomed physical activity, mainly by non-trained subjects, and this process may be of beneficial impact for skeletal muscle adaptation to exercise in initial phases of training. Muscle damage seems to be an important part of muscle hypertrophy (Schoenfeld, 2012), and further investigations should explore the role of native GL levels or GL supplementation in this process.

As recently reported (H.R. Freitas et al., 2015), GL is shown to improve some parameters of sport and exercise performance, and chronic supplementation appears to be of special importance for increasing tolerance to intermittent exercise, lowering feelings of fatigue, and optimizing recovery

from muscle damage. GL may also act as a relevant resource for rehydration during extenuous and prolonged physical activity. In mammalian cells, methylated amines (e.g., betaine) and certain amino acids (e.g., arginine, lysine, taurine, proline and glutamine) are key osmolytes, protecting cells from stressful stimuli (Kidd, Ferket and Garlich, 1997). As previously discussed, these osmolytes are of special interest in competitive sport (Freitas, Barbosa and Ramos, 2015), and the role of GL in hydration should receive additional attention in further research.

## **STRESS AND IMMUNE RESPONSE**

Recent investigations highlights GL as a non-effective supplement for preventing exercise-induced immune impairment (Hoffman et al., 2010), while carbohydrates, fruit/vegetable extracts and quercetin have been reported as modulators of stress response (e.g., diminishing cortisol and epinephrine release) during and after exercise, also, polyphenols, flavonoids and isoquercetin are shown to possess strong anti-inflammatory effects (Nieman, 2013), controlling exacerbated immune response to exercise. As previously discussed, however, GL may act as both metabolic modulator and osmolyte, and some effects of GL supplementation may influence long-term immune adaptations to exercise.

Sasaki et al. (2013) investigated whether chronic GL (3 g/day) supplementation would affect muscle recovery and immune response. Data reveal that GL promoted early recovery from muscle damage and suppression of neutrophil activity, especially in attenuating production of reactive oxygen species (ROS) (Sasaki et al., 2013). Surprisingly, GL is necessary for production of ROS and reactive nitrogen species (RNS) through NADPH in neutrophils. However, GL is also required for glutathione (GSH) synthesis in these cells (Frauwirth, 2014). As previously reported, GSH is exceptionally efficient in neutralizing intracellular ROS, promoting cell survival even under intense cytotoxic environments (Freitas et al., 2016).

Chronic GL ( $\pm$  28 g/day) supplementation did not influence salivary IgA levels in healthy resting athletes, but raised nasal IgA levels during training trials (Krieger et al., 2004). Other study providing lower and acute GL (17.5 g) doses also showed no significant results for salivary IgA levels (Krzykowski et al., 2001). A recent experimental work in mouse model, however, showed that GL supplementation appears to enhance secretory IgA production from mouse

intestine through microbiota, T cell-dependent and independent pathways (Wu et al., 2016).

Two recent studies have explored the role of GL in exercise-induced intestinal permeability. When GL ( $\pm 40$  g or 0.9 g/Kg of free-fat mass) were acutely administered previously to exercise, authors observed attenuation of epithelium permeability and activation of NF- $\kappa$ B pathway in peripheral blood mononuclear cells (PBMCs) (M. Zuhl et al., 2015). Similarly, when high GL doses ( $\pm 48$  g or 0.9 g/Kg of free-fat mass) were provided chronically, data showed decreased production of inflammatory cytokines (through NF- $\kappa$ B activation) and activation of heat shock mechanisms (HSF-1 and HSP70) in PBMCs (M. N. Zuhl et al., 2014). A recent work showed that GL inhibits *in vitro* production of TNF- $\alpha$  and IL-6 from LPS-stimulated human monocytes (Raspé et al., 2013).

## BLOOD MARKERS

Bassini-Cameron et al. (2008) showed that chronic GL (7.08 g/day) supplementation prior to exercise partially prevents hyperammonemia after intermittent and continuous exercise (Bassini-Cameron et al., 2008). Another study from the same group shows that GL supplementation, with or without carbohydrates, is able to decrease 15% overall plasma ammonia levels of highly trained athletes (Carvalho-Peixoto et al., 2007). Balance in GL synthesis is crucial for control of ammonia levels in blood. Specific deletion of mouse liver GL synthetase (GS) increased ammonia concentrations, brain oxidative stress and induced behavior abnormalities (Qvartskhava et al., 2015). While excess GL transport to mitochondria and subsequent hydrolysis may induce ammonia toxicity (Albrecht and Norenberg, 2006), data suggest that the protective effect of GL in ammonia levels of athletes occurs primarily due to a reduction in muscle AMP deamination, as previously described (Walker, Heigenhauser, Hultman and Spriet, 2000).

Johnson et al. (2003) found increased blood and gut mucosa GSH levels after Sprague-Dawley rats were gavaged with 1 g/Kg/day GL (Johnson, Kaufmann, Luo, Todorova and Klimberg, 2003). As previously discussed, GL may be used for GSH synthesis in neutrophils (Frauwirth, 2014), and data suggest that GL is the preferential source for GSH synthesis in mucosal cells of fed piglets (Reeds et al., 1997). Both GL and GSH are associated to protective effects, inhibiting generation of sepsis-induced liver damage in animal models (Babu, Eaton, Drake, Spitz and Pierro, 2001).



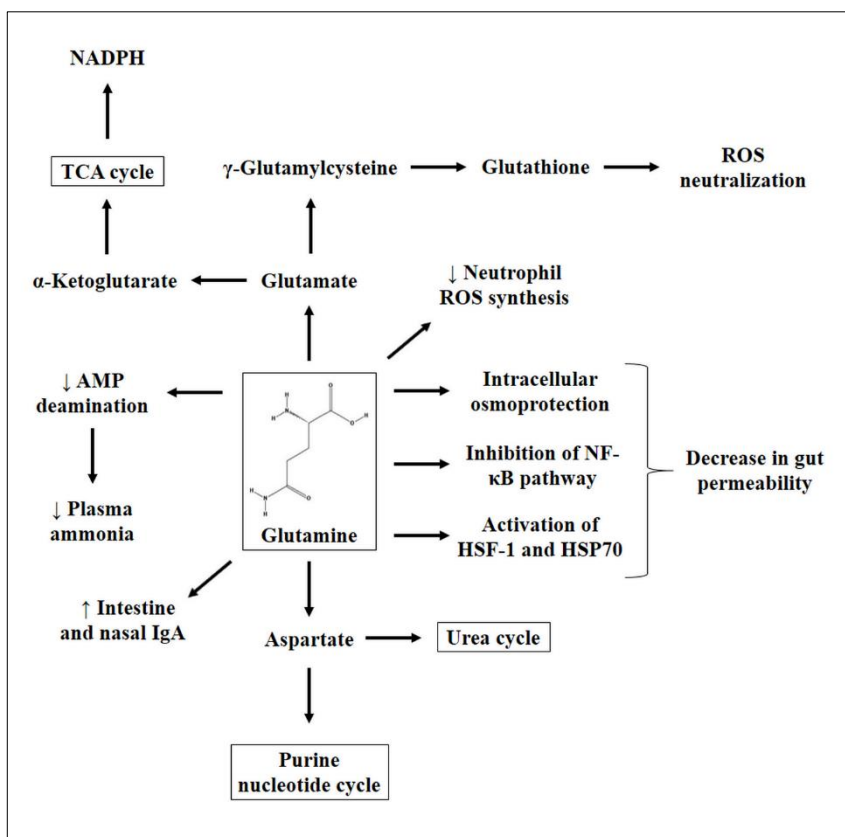


Figure legend: glutamine (GL) is proposed to modulate several intracellular key pathways. GL-induced epithelium osmoprotection, inhibition of NF-κB pathway and activation of heat shock factor 1 (HSF-1) and heat shock protein 70 (HSP70) in peripheral blood mononuclear cells (PBMCs) may decrease intestine permeability, thus reducing endotoxin and systemic inflammation. γ-glutamylcysteine produced from glutamate originates glutathione (GSH), a high-efficiency ROS scavenging low molecular antioxidant. GL may reduce plasma ammonemia by decreasing AMP deamination during extenuous exercise. GL was also shown to decrease neutrophil ROS production and increase intestine and nasal IgA concentrations. GL is also a key substrate for correct functioning of urea, purine nucleotide and tricarboxylic acid (TCA) cycles. PubChem CID: 5961. Adapted from (Nissim, 1999).

Figure 1. Multifactorial role of glutamine in cellular and systemic mechanisms.

Plasma GL levels are itself relevant markers for immune function and effectivity of supplementation. Data shows that enteral GL improves gut permeability status and decreases plasma endotoxin levels of severely burned

patients. Supplementation were correlated to reduced length and costs of hospitalization (Zhou et al., 2015). Lower GL levels were recently associated to cancer-related fatigue observed in patients with systemic inflammation (Schlemmer et al., 2014), and oral GL supplementation seems to attenuate gut permeability, thus diminishing the risk for internalization of endotoxins and general inflammatory status (Yoshida, Kaibara, Ishibashi and Shirouzu, 2001).

Taken together, GL effects on performance may be a consequence of multifactorial cell and systemic mechanisms, including inhibition of inflammatory pathways (e.g., NF- $\kappa$ B activation), accumulation of intracellular osmolytes, synthesis of antioxidants (e.g., GSH), energy metabolism intermediates (e.g.,  $\alpha$ -ketoglutarate), control of global ammonia levels throughout exercise and activation of heat shock response intermediates (e.g., HSF-1 and HSP70). Figure 1 summarizes possible roles of GL in several physiological phenomena ultimately involved in global exercise and physical activity performance.

## REFERENCES

- Albrecht, J., and Norenberg, M. D. (2006). Glutamine: A Trojan horse in ammonia neurotoxicity. *Hepatology*. <http://doi.org/10.1002/hep.21357>.
- Antonio, J., Sanders, M. S., Kalman, D., Woodgate, D., and Street, C. (2002). The Effects of High-Dose Glutamine Ingestion on Weightlifting Performance. *Journal of Strength and Conditioning Research/National Strength and Conditioning Association*, 16(1), 157-160. [http://doi.org/10.1519/1533-4287\(2002\)016<0157:TEOHDG>2.0.CO;2](http://doi.org/10.1519/1533-4287(2002)016<0157:TEOHDG>2.0.CO;2).
- Arwert, L. I., Deijen, J. B., and Drent, M. L. (2003). Effects of an oral mixture containing glycine, glutamine and niacin on memory, GH and IGF-I secretion in middle-aged and elderly subjects. *Nutritional Neuroscience*, 6 (5), 269-275. <http://doi.org/10.1080/10284150310001612195>.
- Babu, R., Eaton, S., Drake, D. P., Spitz, L., and Pierro, A. (2001). Glutamine and glutathione counteract the inhibitory effects of mediators of sepsis in neonatal hepatocytes. In: *Journal of Pediatric Surgery* (Vol. 36, pp. 282-286). <http://doi.org/10.1053/jpsu.2001.20690>.
- Bassini-Cameron, A., Monteiro, A., Gomes, A., Werneck-de-Castro, J. P. S., and Cameron, L. (2008). Glutamine protects against increases in blood ammonia in football players in an exercise intensity-dependent way. *British Journal of Sports Medicine*, 42(November), 260-266. <http://doi.org/10.1136/bjsm.2007.040378>.

- Blomqvist, B., Hammarqvist, F., von der Decken, A., and Wernerman, J. (1995). Glutamine and alpha-ketoglutarate prevent the decrease in muscle free glutamine concentration and influence protein synthesis after total hip replacement. *Metabolism*, 44(9), 1215-1222.
- Cai, G., Yan, J., Zhang, Z., and Yu, Y. (2008). Immunomodulatory effects of glutamine-enriched nutritional support in elderly patients with severe sepsis: A prospective, randomized, controlled study. *Journal of Organ Dysfunction*. Retrieved from <http://ovidsp.ovid.com/ovidweb.cgi?T=JS&CSC=Y&NEWS=N&PAGE=fulltext&D=emed8&AN=2008063742>.
- Carvalho-Peixoto, J., Alves, R. C., and Cameron, L.-C. (2007). Glutamine and carbohydrate supplements reduce ammonemia increase during endurance field exercise. *Applied Physiology, Nutrition, and Metabolism = Physiologie Appliquée, Nutrition et Métabolisme*, 32(6), 1186-90. <http://doi.org/10.1139/H07-091>.
- Castell, L. M., and Newsholme, E. a. (1997). The effects of oral glutamine supplementation on athletes after prolonged, exhaustive exercise. *Nutrition* (Burbank, Los Angeles County, Calif.), 13(7-8), 738-742. [http://doi.org/10.1016/S0899-9007\(97\)83036-5](http://doi.org/10.1016/S0899-9007(97)83036-5).
- Colker, C. M., Swain, M. A., Fabrucini, B., Qiuhi, S., and Kalman, D. S. (2000). Effects of supplemental protein on body composition and muscular strength in healthy athletic male adults. *Current Therapeutic Research - Clinical and Experimental*, 61(1), 19-28. [http://doi.org/10.1016/S0011-393X\(00\)88492-1](http://doi.org/10.1016/S0011-393X(00)88492-1).
- Curthoys, N. P. (2001). Role of mitochondrial glutaminase in rat renal glutamine metabolism. *The Journal of Nutrition*, 131, 2491-2496.
- Cury-Boaventura, M. F., Levada-Pires, A. C., Folador, A., Gorjão, R., Alba-Loureiro, T. C., Hirabara, S. M., ... Pithon-Curi, T. C. (2008). Effects of exercise on leukocyte death: Prevention by hydrolyzed whey protein enriched with glutamine dipeptide. *European Journal of Applied Physiology*, 103(3), 289-294. <http://doi.org/10.1007/s00421-008-0702-1>.
- da Silveira, C. L., de Souza, T. S. P., Batista, G. R., de Araújo, A. T., da Silva, J. C. G., de Sousa, M. do S. C., ... Garrido, N. D. (2014). Is long term creatine and glutamine supplementation effective in enhancing physical performance of military police officers? *Journal of Human Kinetics*, 43, 131-8. <http://doi.org/10.2478/hukin-2014-0098>.
- Favano, A., Santos-Silva, P. R., Nakano, E. Y., Pedrinelli, A., Hernandez, A. J., and Greve, J. M. D. (2008). Peptide glutamine supplementation for tolerance of intermittent exercise in soccer players. *Clinics* (Sao Paulo, Brazil), 63(1), 27-32. <http://doi.org/10.1590/S1807-59322008000100006>.

- Frauwirth, K. (2014). Glutamine Uptake and Immunomodulation: An Overview. *Nutrition and Health*, 55-66.
- Freitas, H. R., Barbosa, M. R., and Ramos, T. da S. (2015). O papel da suplementação de betaina na atividade física: uma revisão sistemática [The role of betaine supplementation on physical activity: a systematic review]. *Nutrire*, 40(2), 246-260. <http://doi.org/10.4322/2316-7874.50914>.
- Freitas, H. R., da Silva Pereira, A., and da Silva Ramos, T. (2015). The effects of acute/chronic glutamine and glutamine peptide supplementation on the performance and immune function in young active adult athletes. *Current Nutrition and Food Science*, 11(4), 315-322.
- Freitas, H. R., Ferraz, G., Ferreira, G. C., Ribeiro-Resende, V. T., Chiarini, L. B., Nascimento, J. L. M. D., ... De Melo Reis, R. A. (2016). Glutathione-induced calcium shifts in chick retinal glial cells. *PLoS ONE*, 11(4). <http://doi.org/10.1371/journal.pone.0153677>.
- Galera, S. C., Fachine, F. V., Teixeira, M. J., Coelho, Z. C. B., de Vasconcelos, R. C., and Leitão de Vasconcelos, P. R. (2010). The safety of oral use of l-glutamine in middle-aged and elderly individuals. *Nutrition*, 26(4), 375-381. <http://doi.org/10.1016/j.nut.2009.05.013>.
- Gauthier, S. F., Pouliot, Y., and Saint-Sauveur, D. (2006). Immunomodulatory peptides obtained by the enzymatic hydrolysis of whey proteins. *International Dairy Journal*. <http://doi.org/10.1016/j.idairyj.2006.06.014>.
- Gleeson, M., and Bishop, N. C. (2000). Modification of immune responses to exercise by carbohydrate, glutamine and anti-oxidant supplements. *Immunology and Cell Biology*. <http://doi.org/10.1046/j.1440-1711.2000.00953.x>.
- Halson, S. L., Lancaster, G. I., Achten, J., Gleeson, M., and Jeukendrup, A. E. (2004). Effects of carbohydrate supplementation on performance and carbohydrate oxidation after intensified cycling training. *Journal of Applied Physiology* (Bethesda, Md. : 1985), 97(4), 1245-1253. <http://doi.org/10.1152/jappphysiol.01368.2003>.
- Hiscock, N., Petersen, E. W., Krzykowski, K., Boza, J., Halkjaer-Kristensen, J., and Pedersen, B. K. (2003). Glutamine supplementation further enhances exercise-induced plasma IL-6. *Journal of Applied Physiology* (Bethesda, Md. : 1985), 95(1), 145-8. <http://doi.org/10.1152/jappphysiol.00471.2002>.
- Hoffman, J. R., Ratamess, N. a, Kang, J., Rashti, S. L., Kelly, N., Gonzalez, A. M., ... Maresh, C. M. (2010). Examination of the efficacy of acute L-alanyl-L-glutamine ingestion during hydration stress in endurance exercise. *Journal of the International Society of Sports Nutrition*, 7, 8. <http://doi.org/10.1186/1550-2783-7-8>.

- Hoffman, J. R., Williams, D. R., Emerson, N. S., Hoffman, M. W., Wells, A. J., McVeigh, D. M., ... Fragala, M. S. (2012). L-alanyl-L-glutamine ingestion maintains performance during a competitive basketball game. *Journal of the International Society of Sports Nutrition*, 9(1), 4. <http://doi.org/10.1186/1550-2783-9-4>.
- Ionescu, A. M., Vasilescu, M. M., Carmoci, A., Nica, A. S., and Ionescu, S. (2014). Long-term glutamine supplementation in elite gymnasts. *Farmacia*, 62(4), 761-766.
- Johnson, A. T., Kaufmann, Y. C., Luo, S., Todorova, V., and Klimberg, V. S. (2003). Effect of glutamine on glutathione, IGF-I, and TGF- $\beta$ 1. In: *Journal of Surgical Research* (Vol. 111, pp. 222-228). [http://doi.org/10.1016/S0022-4804\(03\)00083-0](http://doi.org/10.1016/S0022-4804(03)00083-0).
- Kaldirimci, M., Sajedi, H., Sam, C., Mizrak, O., and Kavurmaci, H. (2015). Glutamine Supplementation and Basketball Players Power Performance Changes. *Journal of Sports Science*, 3, 298-304.
- Keast, D., Arstein, D., Harper, W., Fry, R., and Morton, A. (1995). Depression of plasma glutamine concentration after exercise stress and its possible influence on the immune system. *Medical Journal of Australia*, 162(1), 15-18.
- Khorshidi-Hosseini, M., and Nakhostin-Roohi, B. (2013). Effect of glutamine and maltodextrin acute supplementation on anaerobic power. *Asian Journal of Sports Medicine*, 4(2), 131-136.
- Kidd, M. T., Ferket, P. R., and Garlich, J. D. (1997). Nutritional and osmoregulatory functions of betaine. *World's Poultry Science Journal*, 53 (02), 125-139. <http://doi.org/doi:10.1079/WPS19970013>.
- Krieger, J. W., Crowe, M., and Blank, S. E. (2004). Chronic glutamine supplementation increases nasal but not salivary IgA during 9 days of interval training. *Journal of Applied Physiology* (Bethesda, Md.: 1985), 97 (2), 585-591. <http://doi.org/10.1152/japplphysiol.00971.2003>.
- Krzywkowski, K., Petersen, E. W., Ostrowski, K., Link-Amster, H., Boza, J., Halkjaer-Kristensen, J., and Pedersen, B. K. (2001). Effect of glutamine and protein supplementation on exercise-induced decreases in salivary IgA. *Journal of Applied Physiology* (Bethesda, Md.: 1985), 91(2), 832-8. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/11457800>.
- Kuhn, K. S., Muscaritoli, M., Wischmeyer, P., and Stehle, P. (2010). Glutamine as indispensable nutrient in oncology: Experimental and clinical evidence. *European Journal of Nutrition*. <http://doi.org/10.1007/s00394-009-0082-2>.
- Legault, Z., Bagnall, N., and Kimmerly, D. S. (2015). The influence of oral L-glutamine supplementation on muscle strength recovery and soreness

- following unilateral knee extension eccentric exercise. *International Journal of Sport Nutrition and Exercise Metabolism*, 25(5), 417-426. <http://doi.org/10.1123/ijsnem.2014-0209>.
- López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M., and Kroemer, G. (2013). The hallmarks of aging. *Cell*. <http://doi.org/10.1016/j.cell.2013.05.039>.
- Newsholme, E., Newsholme, P., and Curi, R. (1987). The role of the citric acid cycle in cells of the immune system and its importance in sepsis, trauma and burns. *Biochemical Society Symposia*, 54, 145-162.
- Newsholme, P. (2001). Why is L-glutamine metabolism important to cells of the immune system in health, postinjury, surgery or infection? *The Journal of Nutrition*, 131, 2515S-22S; discussion 2523S-4S.
- Newsholme, P., Curi, R., Gordon, S., and Newsholme, E. A. (1986). Metabolism of glucose, glutamine, long-chain fatty acids and ketone bodies by murine macrophages. *The Biochemical Journal*, 239(1), 121-5. Retrieved from <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1147248&tool=pmcentrez&rendertype=abstract>.
- Newsholme, P., Curi, R., Pithon-Curi, T. C., Murphy, C. J., Garcia, C., and Pires De Melo, M. (1999). Glutamine metabolism by lymphocytes, macrophages, and neutrophils: Its importance in health and disease. *Journal of Nutritional Biochemistry*. [http://doi.org/10.1016/S0955-2863\(99\)00022-4](http://doi.org/10.1016/S0955-2863(99)00022-4).
- Nieman, D. (2013). Exercise, Nutrition, and Immune Function. *John Wiley and Sons*, 19, 478-489.
- Nissim, I. (1999). Newer aspects of glutamine/glutamate metabolism: the role of acute pH changes. *The American Journal of Physiology*, 277(4 Pt 2), F493-7. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/10516271>.
- Pruna, G. J., Hoffman, J. R., McCormack, W. P., Jajtner, A. R., Townsend, J. R., Bohner, J. D., ... Fukuda, D. H. (2014). Effect of acute L-Alanyl-L-Glutamine and electrolyte ingestion on cognitive function and reaction time following endurance exercise. *European Journal of Sport Science*, 1391 (March 2015), 1-8. <http://doi.org/10.1080/17461391.2014.969325>.
- Qvartskhava, N., Lang, P. a., Görg, B., Pozdeev, V. I., Ortiz, M. P., Lang, K. S., ... Häussinger, D. (2015). Hyperammonemia in gene-targeted mice lacking functional hepatic glutamine synthetase. *Proceedings of the National Academy of Sciences*, 112(17), 201423968. <http://doi.org/10.1073/pnas.1423968112>.
- Ramallo, B., Charro, M. A., Foschini, D., Prestes, J., Pithon-Curi, T., Evangelista, A., ... Galatti, L. (2013). ACUTE Glutamine Supplementation Does not Affect Muscle Damage Profile after Resistance Training.

- International Journal of Sports Science*. <http://doi.org/10.5923/j.sports.20130301.02>.
- Raspé, C., Czeslick, E., Weimann, A., Schinke, C., Leimert, A., Kellner, P., ... Sablotzki, A. (2013). Glutamine and alanine-induced differential expression of intracellular IL-6, IL-8, and TNF- $\alpha$  in LPS-stimulated monocytes in human whole-blood. *Cytokine*, 62(1), 52-57. <http://doi.org/10.1016/j.cyto.2013.02.020>.
- Reeds, P. J., Burrin, D. G., Stoll, B., Jahoor, F., Wykes, L., Henry, J., and Frazer, M. E. (1997). Enteral glutamate is the preferential source for mucosal glutathione synthesis in fed piglets. *Am. J. Physiol.*, 273(2 Pt 1), E408-15.
- Rowbottom, D. G., Keast, D., and Morton, A. R. (1996). The emerging role of glutamine as an indicator of exercise stress and overtraining. *Sports Medicine* (Auckland, N.Z.), 21(2), 80-97. <http://doi.org/10.2165/00007256-199621020-00002>.
- Sasaki, E., Umeda, T., Takahashi, I., Arata, K., Yamamoto, Y., Tanabe, M., ... Nakaji, S. (2013). Effect of glutamine supplementation on neutrophil function in male judoists. *Luminescence*, 28(4), 442-449. <http://doi.org/10.1002/bio.2474>.
- Schlemmer, M., Suchner, U., Schäpers, B., Duerr, E. M., Alteheld, B., Zwingers, T., ... Zimmer, H. G. (2014). Is glutamine deficiency the link between inflammation, malnutrition, and fatigue in cancer patients? *Clinical Nutrition*. <http://doi.org/10.1016/j.clnu.2014.12.021>.
- Schoenfeld, B. J. (2012). Does exercise-induced muscle damage play a role in skeletal muscle hypertrophy? *Journal of Strength and Conditioning Research/National Strength and Conditioning Association*, 26(5), 1441-53. <http://doi.org/10.1519/JSC.0b013e31824f207e>.
- Steensberg, A., Keller, C., Starkie, R. L., Osada, T., Febbraio, M. a, and Pedersen, B. K. (2002). IL-6 and TNF-alpha expression in, and release from, contracting human skeletal muscle. *American Journal of Physiology. Endocrinology and Metabolism*, 283(6), E1272-E1278. <http://doi.org/10.1152/ajpendo.00255.2002>.
- Walker, J. L., Heigenhauser, G. J., Hultman, E., and Spriet, L. L. (2000). Dietary carbohydrate, muscle glycogen content, and endurance performance in well-trained women. *Journal of Applied Physiology* (Bethesda, Md. : 1985), 88(6), 2151-2158. Retrieved from <http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=10846030&retmode=ref&cmd=prlinks&npapers2://publication/uuid/BFD13BB5-BDA1-4EE9-B0BE-70BFCDE9FAFA>.

- Walsh, N. P., Blannin, A. K., Robson, P. J., and Gleeson, M. (1998). Glutamine, exercise and immune function: Links and possible mechanisms. *Sports Medicine*. <http://doi.org/10.2165/00007256-199826030-00004>.
- Wu, M., Xiao, H., Liu, G., Chen, S., Tan, B., Ren, W., ... Yin, Y. (2016). Glutamine promotes intestinal SIgA secretion through intestinal microbiota and IL-13. *Molecular Nutrition and Food Research*. <http://doi.org/10.1002/mnfr.201600026>.
- Yoshida, S., Kaibara, a, Ishibashi, N., and Shirouzu, K. (2001). Glutamine supplementation in cancer patients. *Nutrition* (Burbank, Los Angeles County, Calif.), 17(9), 766-768. [http://doi.org/10.1016/S0899-9007\(01\)00629-3](http://doi.org/10.1016/S0899-9007(01)00629-3).
- Zhou, Y.-P., Jiang, Z.-M., Sun, Y.-H., Wang, X.-R., Ma, E.-L., and Wilmore, D. (2015). The effect of supplemental enteral glutamine on plasma levels, gut function, and outcome in severe burns: a randomized, double-blind, controlled clinical trial. *JPEN. Journal of Parenteral and Enteral Nutrition*, 27(4), 241-5. <http://doi.org/10.1177/0148607103027004241>.
- Zuhl, M., Dokladny, K., Mermier, C., Schneider, S., Salgado, R., and Moseley, P. (2015). The effects of acute oral glutamine supplementation on exercise-induced gastrointestinal permeability and heat shock protein expression in peripheral blood mononuclear cells. *Cell Stress and Chaperones*, 20(1), 85-93. <http://doi.org/10.1007/s12192-014-0528-1>.
- Zuhl, M. N., Lanphere, K. R., Kravitz, L., Mermier, C. M., Schneider, S., Dokladny, K., and Moseley, P. L. (2014). Effects of oral glutamine supplementation on exercise-induced gastrointestinal permeability and tight junction protein expression. *Journal of Applied Physiology*, 116(2), 183-191. <http://doi.org/10.1152/jappphysiol.00646.2013>.

## BIOGRAPHICAL SKETCH

Bachelor of Science in Human Nutrition at the Federal University of the State of Rio de Janeiro, Specialist in Phytotherapy at AVM Integrated Faculty, Master of Science in Biological Sciences (Biophysics/Neurobiology), Licentiate in Biological Sciences at Salgado de Oliveira University and Philosophiae Doctor graduate at the Federal University of Rio de Janeiro, recipient of research fellowships from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (2015-2016) and from Conselho Nacional de Desenvolvimento Científico e Tecnológico (2016-). Author of research/review articles in national



and international peer-reviewed journals, such as “Journal of Nutrition and Health Sciences,” “Current Nutrition and Food Science” and “PLoS One,” including one “Editor’s Choice Article” (Current Nutrition and Food Science, 11(4): 315-322). Author of the textbook “Principles of Bioethics” (Salgado de Oliveira University Press). Invited reviewer at “Current Nutrition and Food Science” (Bentham Science Publishers), “IMPULSE - Premier Neuroscience Journal” (Appalachian State University) and International Research Journal of Medicine and Medical Sciences (NetJournals). Associate editor at Food and Nutrition (Lawarence Press). Invited lecturer in the discipline of research methodology at Federal University of the State of Rio de Janeiro and neurophysiology at Federal University of Rio de Janeiro. Founder and supervisor of the academic league of sports nutrition and the academic league of clinical nutrition at Federal University of the State of Rio de Janeiro. Interested in pharmacological mechanisms of dietary supplements, neuroglial communication, brain’s antioxidant mechanisms, cannabinoid system, n-3 and n-6 fatty acids metabolism and feeding behavior. Research expertise in fluorescence microscopy, immunoassays and cell culture. Member of the Biochemical Society (London/UK) since 2014.

# INDEX

## A

- acetaminophen, 84
- acetonitrile, 142
- acid, xi, xiii, 3, 29, 34, 41, 45, 52, 66, 68, 73, 74, 75, 116, 117, 119, 120, 125, 126, 128, 137, 138, 142, 143, 147, 189, 194, 197, 201, 202, 207, 208, 218, 223
- acidic, xi, 115, 116, 117, 118, 119, 120, 122, 125
- acridine orange (AO), x, 62, 72
- active compound, 160
- acute leukemia, 201
- acute lung injury, 90
- adaptation(s), 195, 215, 216
- adaptive immune response, ix, 32
- adenine, x, 62, 63, 64, 72
- adenine nucleotide translocase (ANT), x, 62, 63, 64, 76
- adenocarcinoma, 192
- adenosine, x, 62, 68, 73
- adenosine triphosphate (ATP), x, 62
- adenovirus, 96, 97, 98, 100, 103, 105, 108, 109, 110, 111, 112, 113
- adjunctive therapy, 35
- ADP, 68, 70
- adults, 33, 51, 191, 195, 203, 220
- advancement, 123
- adverse effects, 3, 163, 174, 175, 185, 194
- age-related diseases, 145
- aggregation, viii, 2, 4, 6, 7, 21
- aging process, 145
- alanine, 190, 224
- alcohol consumption, 156
- algae, 142
- algorithm, 34
- allele, 7, 107
- allergy, 181
- aloe, 171
- amenorrhea, 156
- American Heart Association, 47, 48
- amines, 216
- amino acid(s), xiii, 3, 136, 189, 201, 202, 207, 209, 214, 216
- ammonia, xiii, 207, 211, 217, 219
- amorphous polymers, 124
- amygdala, 3
- androgen, 157, 164, 166
- anemia, 192
- angiogenesis, 45, 146
- antagonism, 146
- antibody, 39, 44, 53
- anticancer drug, 198
- antigen, ix, 32, 45, 97
- antigen-presenting cells, ix, 32
- antioxidant, xiii, 9, 18, 23, 66, 74, 75, 77, 81, 85, 86, 133, 134, 138, 143,

148, 189, 190, 195, 196, 199, 200,  
201, 202, 218, 226

antisense, viii, 32, 34, 39, 53

antisense oligonucleotides, viii, 32, 34

antitumor, x, 56, 95, 97, 101, 108, 110

anxiety, 155

aorta, 43, 44, 58

apoptosis, xiii, 5, 9, 11, 12, 15, 24, 45,  
63, 66, 68, 70, 76, 78, 79, 84, 86, 87,  
101, 103, 136, 189, 190, 196, 200,  
202, 204, 208, 209

arginine, 10, 203, 216

arteriolar smooth muscle cells (ASMC),  
x, 62, 69, 78

artery, 33

ASN, viii, 1, 2, 3, 4, 6, 7, 14, 16

aspartate, 77

assessment, xi, 35, 70, 71, 72, 96, 98,  
106, 107, 108, 165, 167

astrocytes, 200

ataxia, 203

ataxiatangiectasia mutated (ATM), 96,  
102, 104

atherogenesis, 35, 42, 49, 57

Atherosclerosis, viii, ix, 31, 32, 33, 36,  
41, 47, 48, 49, 50, 54, 55, 56, 155

atherosclerotic plaque, 36

athletes, 209, 210, 215, 216, 217, 220,  
221

ATP, x, 6, 15, 34, 35, 62, 63, 66, 67, 68,  
70, 71, 72, 73, 74, 76, 197

atractyloside (ATR), x, 62, 68

atrial fibrillation, 175

atrophic vaginitis, 154

atrophy, 5, 29, 154, 157, 159, 165, 196

autonomic nervous system, 3

Autophagy, v, ix, 31, 32, 40, 41, 55, 56,  
57, 85

autosomal dominant, 9, 11, 12, 14, 22,  
28, 29

autosomal recessive, 4, 10, 11, 18, 19,  
23, 24

avascular necrosis, 187

<b>B</b>
----------

back pain, 155

basal ganglia, 3, 5, 29

base, 29, 42, 72, 110, 113, 149, 201

beneficial effect, 73, 140, 147, 148, 161,  
195

benefits, 34, 48, 145, 159, 162, 163, 183,  
184, 185, 191, 213

beta-glucans, ix, 32, 34, 35, 38

Bioactive, v, 115, 149

bioactive agents, vii, 116

bioavailability, 77, 141

biochemistry, 146

biological activity, 134, 137, 143

biological fluids, xi, 115

biomarkers, 16, 21, 36, 50, 213

biosynthesis, viii, 2, 4, 37, 190

birth control, 158

bisphosphonates, xii, xiii, 173, 174, 175,  
176, 177, 178, 179, 180, 182, 183,  
184, 185, 186, 187

bladder cancer, 113

bleeding, 154, 158, 159, 164, 165, 168

blood, xi, 3, 7, 22, 39, 43, 50, 51, 62, 65,  
84, 88, 96, 98, 107, 108, 113, 139,  
154, 155, 156, 163, 176, 180, 217,  
218, 219, 224, 225

blood clot, 163

blood flow, 65, 176

blood monocytes, 84

blood plasma, 139

blood pressure, 3

blood supply, 180

blood transfusion, 62

blood-brain barrier, 3

body composition, 205, 220

body weight, 44, 212

bone, 111, 174, 175, 176, 178, 179, 183,  
184, 185, 187, 192, 193, 199, 202

bone marrow, 199

bone mass, 174

bone resorption, 174, 184

bones, xii, 158, 173, 176, 180

bounds, 184

brain, 3, 8, 15, 17, 22, 27, 28, 42, 81, 88,  
107, 113, 196, 198, 200, 204, 217,  
226  
brain stem, 3, 8  
brain tumor, 113  
brainstem, 5, 7  
Brazil, 207, 220  
breast cancer, 113, 146, 152, 158, 162,  
163, 164, 167, 168  
BRONJ, 174, 175

## C

Ca<sup>2+</sup>, x, 61, 64, 67, 70, 73, 75  
cachexia, 196  
cadmium, 43, 85  
caffeine, xi, 116, 117, 118, 119, 120,  
122, 125, 128  
calcification, 67  
calcium, 36, 63, 67, 74, 75, 76, 77, 82,  
196, 200, 221  
cancer, vii, x, 23, 96, 97, 98, 99, 100,  
101, 102, 104, 105, 106, 107, 108,  
109, 110, 111, 112, 113, 146, 156,  
158, 159, 163, 164, 167, 168, 175,  
176, 177, 183, 184, 186, 191, 192,  
194, 195, 196, 197, 199, 200, 201,  
202, 204, 208, 219, 224, 225  
cancer cells, 99, 100, 101, 102, 104, 105,  
106, 107, 112, 195, 197, 202  
cancer therapy, 109, 186  
cancerous cells, 196, 199  
candidates, 53, 139, 214  
capillary, xi, 116, 137  
carbohydrate, 210, 211, 220, 221, 224  
carbohydrates, 197, 209, 211, 213, 214,  
215, 216, 217  
carcinogenesis, 41, 87  
carcinoma, 56, 174, 175  
cardiac arrest, 83  
cardiac output, 65, 81  
cardiovascular disease, vii, viii, ix, 31,  
32, 33, 48, 52, 54, 62, 155, 157  
cardiovascular risk, viii, 32, 48, 54, 167  
catabolism, 40, 41, 54

catalase (CAT), x, 62, 74, 195  
catecholamines, 204  
catheter, 89  
Caucasian population, 10  
cDNA, 38, 198  
cell biology, 27, 63, 81  
cell culture, 226  
cell cycle, x, 5, 95, 99, 100  
cell death, x, 4, 15, 25, 46, 81, 82, 84,  
88, 95, 97, 98, 99, 101, 102, 103, 104,  
108, 111, 190, 215  
cell division, 99, 100  
cell killing, 82  
cell line, 87, 101, 105, 107, 202  
cell membranes, 138  
cell metabolism, 63, 76, 203  
cell signaling, 7, 43  
cell surface, 97  
cellular regulation, 134  
cellulose, 117, 130  
central nervous system (CNS), vii, 1, 2  
cerebral edema, xiv, 208  
cervix, 107, 154  
chelates, 78  
chemical, xi, 36, 71, 98, 110, 116, 118,  
132, 133, 134, 139, 158, 161, 169  
chemical bonds, 71  
chemical characteristics, 116  
chemical reactions, 158  
chemical structures, 36, 132, 134  
chemicals, 146, 160  
chemotherapeutic agent, 101, 112  
chemotherapy, xi, 96, 98, 101, 103, 105,  
108, 177, 183, 190, 194, 196, 197,  
201, 208  
children, 38, 52, 152, 191, 197, 201, 204  
China, 61, 89, 90, 91, 92, 159  
chitosan, 52  
cholecystitis, 158  
cholestasis, 44, 58  
cholesterol, viii, ix, 31, 32, 33, 34, 35,  
36, 37, 38, 39, 40, 41, 46, 49, 50, 51,  
52, 53, 54, 56  
chromatography, 139, 142  
chromosome, 24, 27, 99

- CID, 218  
 circulating tumor cells (CTC), xi, 96, 98,  
     107, 111, 112, 113  
 circulation, 3, 39  
 cirrhosis, 39, 41, 56  
 citrulline, 190, 196  
 clinical application, x, 62, 104  
 clinical symptoms, xiii, 173, 183  
 clinical trials, 40, 48, 80, 161, 162  
 CNS, vii, 1, 2, 3, 15, 16  
 coenzyme, 37, 52, 73, 74  
 cognitive function, 157, 166, 200, 223  
 cognitive impairment, 12, 14  
 collagen, xii, 44, 45, 131, 136, 137, 138,  
     140, 157, 180  
 colon, 107, 195, 199  
 colonization, 112, 180  
 colorectal cancer, 107, 111  
 combination therapy, x, 96, 98, 105, 108  
 comparative analysis, 28  
 compatibility, 124  
 competition, 119, 213  
 competitive advantage, 213  
 competitive sport, 208, 216  
 complement, 16  
 complications, 14, 42, 174, 196, 199  
 composition, 55, 78, 142, 205, 220  
 compounds, ix, xi, 15, 32, 34, 35, 36, 37,  
     39, 43, 73, 75, 116, 118, 119, 121,  
     122, 131, 132, 133, 134, 135, 142,  
     143, 152, 160, 163  
 condensation, 178  
 conditionally essential, 190  
 conductance, 63  
 configuration, 133  
 conformational diseases, 21  
 congenital heart disease, 158  
 connective tissue, 148  
 consumption, 65, 66, 156, 166, 170, 210  
 control group, 5, 38, 70  
 controlled release, v, vii, 115, 116, 117,  
     129, 130  
 controlled trials, 48, 169, 171, 191  
 copper, 11, 36  
 coronary artery disease, 33  
 coronary heart disease, viii, 31, 34, 35,  
     36, 54, 157, 167  
 cortex, 29  
 cortical neurons, 12  
 corticosteroids, 176  
 cortisol, 195, 213, 216  
 cosmetic, xi, 131, 132, 133, 137, 138,  
     139, 141, 142, 143, 146, 148, 167  
 costs of production, 142  
 coxsackie and adenovirus receptor  
     (CAR), 96, 97  
 CPC, 195  
 creatine, 44, 220  
 creatinine, 214  
 CRIT, 90  
 cross-sectional study, 166  
 crystallization, 41, 54  
 crystals, 40, 69  
 CSF, 44  
 cultivation, 180  
 culture, 137, 138, 147, 226  
 CVD, vii, ix, 32, 33, 34, 35, 37, 38, 40,  
     46, 54  
 cycles, 153, 154, 218  
 cyclic adenosine monophosphate  
     (cAMP), x, 62, 73  
 cycling, 221  
 cyclodextrins, 141  
 cyclooxygenase, 97  
 cyclophilin D (CyPD), x, 62, 64, 75, 79  
 cyclosporine A (CsA), x, 62, 69, 72, 75  
 cyst, 192  
 cysteine, 15, 42, 43, 45, 46, 56, 58, 75,  
     198  
 cytochrome, 68, 70, 73, 79, 83, 84, 190  
 cytokines, xii, xiii, 40, 44, 45, 46, 59, 78,  
     131, 138, 197, 208, 217  
 cytometry, 71, 138  
 cytoplasm, 76  
 cytoskeleton, 190  
 cytotoxicity, 9, 11, 24, 148, 198

## D

deacetylation, 76, 78, 83, 87, 134

deaths, 33  
 decomposition, 63, 137, 143  
 defects, 5, 12, 184  
 deficiency, 7, 42, 54, 57, 63, 71, 136,  
 140, 156, 157, 199, 224  
 degradation, xii, 3, 4, 5, 6, 9, 10, 11, 19,  
 20, 39, 102, 104, 117, 131, 138, 157,  
 190  
 dementia, 3, 5, 7, 14, 21  
 dentist, 174, 182, 185  
 depolarization, 67, 71  
 depression, ix, 32, 40, 42, 43, 44, 45, 46,  
 47, 58, 155  
 deprivation, 195, 202, 203  
 derivatives, 129, 136, 142, 170  
 dermis, 136, 137, 143  
 desorption, 120  
 detectable, 105  
 detection, x, 33, 96, 98, 99, 107, 108,  
 110, 112, 113, 143, 165  
 detection system, x, 96, 98, 99, 107, 110  
 detoxification, 204  
 deviation, 122, 123  
 diabetes, 33, 48, 51, 52, 56, 62, 176, 177  
 diabetic patients, 37  
 diet, 37, 38, 44, 50, 51, 135, 144, 159,  
 162, 197, 200, 205, 209  
 dietary fiber, 38, 51, 52  
 dietary supplementation, 37, 38, 52  
 diffusion, xi, 70, 116, 118, 119, 120,  
 123, 124, 127, 128, 129, 135  
 diffusion mechanisms, xi, 116  
 digestion, 160  
 dihydroxyphenylalanine, 15  
 dimensionality, 129  
 disability, 62  
 discomfort, 157  
 disease gene, 22  
 disease progression, 42  
 diseases, vii, viii, ix, xiii, 1, 9, 16, 17,  
 21, 31, 32, 43, 62, 145, 152, 154, 155,  
 159, 170, 174, 176, 177, 183, 185,  
 187, 189, 194, 208, 214  
 disorder, 156, 159, 176  
 dissociation, 64, 117

distilled water, xi, 116, 118, 119, 120,  
 122, 123, 125  
 distress, 165  
 distribution, 51, 67, 81  
 diversity, 143  
 dizziness, 155  
 DJ-1, viii, 2, 4, 8, 9, 11, 16, 23, 24, 190,  
 201  
 DNA, 5, 15, 38, 96, 101, 104, 107, 112,  
 136, 190, 197, 200  
 DNA damage, 5, 15, 101, 104, 200  
 DNA double strand-breaks (DSBs), 96,  
 101  
 DNA repair, 104, 112, 190  
 dopamine, viii, 2, 17  
 dopaminergic, 2, 4, 6, 13, 15, 17, 24  
 dosage, 116, 128, 180  
 down-regulation, 83  
*Drosophila*, 28  
 drug delivery, 110, 128, 130  
 drug release, 129  
 drug resistance, 198, 204  
 drug treatment, 17  
 drugs, x, 35, 36, 61, 77, 83, 129, 174,  
 182, 183, 184, 185, 194, 196, 198,  
 199  
 dyslipidemia, 50, 53  
 dystonia, 5, 10

## E

elastin, xii, 131, 136, 138, 140  
 electrolyte, 122, 210, 223  
 electron, x, 43, 62, 66, 70, 73, 75  
 electron microscopy, 43  
 electron transport system (ETS), x, 62,  
 66, 73  
 electron-transferring flavin proteins  
 (ETFs), x, 62, 73  
 elongation, 99, 100  
 embryonic stem cells, 86  
 emotional intelligence, 155  
 encoding, 3, 7, 21, 25  
 endocrine, 133, 135, 152, 213

endocrine system, 133, 135  
 endocrinology, 168, 170  
 endometrial hyperplasia, 163  
 endometriosis, 156  
 endosperm, 117  
 endothelial cells, 204  
 endothelial dysfunction, 164  
 endotoxins, 219  
 endurance, 51, 211, 214, 220, 221, 223, 224  
 energy, vii, x, xiii, 61, 62, 63, 66, 69, 77, 138, 155, 207, 213, 219  
 engineering, 98  
 England, 19, 164, 167  
 enlargement, 154  
 entanglements, 124  
 environment, 77, 121, 125, 184  
 environmental factors, 33, 136, 156  
 environments, 33, 216  
 enzymatic activity, 10, 87, 139  
 enzyme, xii, 5, 7, 9, 36, 46, 59, 67, 71, 72, 73, 74, 75, 78, 79, 84, 99, 131, 134, 138, 145, 190, 199  
 epidemic, 187  
 epidemiologic, 167  
 epidemiology, 167  
 epidermal growth factor receptor (EGFR), 96, 101, 103  
 epidermis, 136, 139  
 epigenetic alterations, 214  
 epinephrine, 216  
 epithelial cells, 68, 76, 78, 88, 196, 203  
 epithelial ovarian cancer, 163  
 epithelium, 136, 170, 197, 203, 217, 218  
 equilibrium, 118  
 erosion, 116, 118, 119, 120, 122, 123, 124, 125, 126, 128, 130  
 erythrocytes, 190, 200  
 esophageal cancer, 105  
 esophagus, 107, 174  
 ester, viii, 32, 34, 39, 51, 54, 75  
 estriol, 135  
 estrogen, xi, 131, 132, 133, 134, 135, 136, 140, 145, 146, 148, 153, 154,

156, 157, 158, 159, 160, 163, 164, 167, 168, 169, 170  
 estrogen receptor modulator, 133, 145, 148  
 ethanol, 87, 143, 202  
 eukaryotic cell, 5, 67  
 evidence, xii, 14, 22, 29, 45, 64, 76, 80, 108, 152, 159, 161, 162, 167, 169, 170, 184, 191, 213, 222  
 examinations, 186  
 excretion, 37, 52  
 exercise, vii, xiii, 33, 50, 161, 170, 207, 208, 209, 210, 211, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225  
 exercise performance, 215  
 exocytosis, 12, 13  
 exons, 4, 13  
 experimental condition, 138  
 expertise, 144, 226  
 exposure, xi, 116, 137, 166, 190  
 extraction, 65, 142, 149, 175, 178, 179, 182, 183, 186  
 extracts, 141, 142, 149, 162, 169, 216

## F

fabrication, xi, 115  
 familial hypercholesterolemia, 38, 39, 53  
 families, 7, 8, 12, 18, 21, 23  
 family members, 13, 76  
 fat, 38, 41, 51, 52, 211, 217  
 fatty acids, 34, 73, 117, 138, 191, 201, 223, 226  
 feces, 37  
 femur, 192  
 fertility, 164, 195  
 fiber, 36, 37, 38, 51, 52, 136, 137, 140, 142, 143  
 fibrillation, 6, 175  
 fibroblasts, 5, 18, 136, 137, 138, 143, 146, 203  
 fibrogenesis, 44  
 fibrosis, 41, 42, 44, 45, 46, 57, 58  
 fistulas, 178, 181, 184

flavonoids, 133, 160, 216  
 flavor, x, 62, 73  
 flexibility, 211  
 flotation, 123  
 flour, 133, 142, 149  
 fluctuations, 15, 29  
 fluid, 75, 85, 140, 149, 210  
 fluid extract, 149  
 fluorescence, x, 71, 84, 96, 98, 105, 106, 143, 226  
 fluorophores, 82  
 follicle, 154, 156, 164  
 follicle stimulating hormone, 156  
 follicles, 154  
 food, xii, 51, 129, 133, 159, 173, 185  
 Food and Drug Administration FDA, xi, 108, 115  
 formation, xi, 3, 6, 7, 10, 13, 14, 16, 20, 27, 39, 40, 41, 46, 57, 67, 74, 116, 117, 121, 128, 137, 147, 149, 178, 179, 182  
 formula, 139  
 fractures, 175, 178  
 frameshift mutation, 10  
 free radicals, xii, 66, 78, 131, 138, 199  
 functional capillaries density (FCD), x, 62, 65  
 functional changes, 10

## G

gadolinium, ix, 32, 40, 42, 43, 45, 46, 47, 57, 58, 59  
 gallbladder disease, 159  
 ganglion, 193  
 gastrointestinal tract, 116, 196  
 GdCl<sub>3</sub>, ix, 32, 43, 44, 45, 46, 58, 59  
 gel, xi, 36, 115, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 130  
 gel formation, 117  
 gene expression, 54, 97, 136  
 gene therapy, 110  
 gene transfer, 98, 108  
 general anaesthesia, 89  
 genes, viii, 2, 3, 5, 9, 14, 16, 20, 22, 97, 99, 101, 102, 106, 107, 135, 155, 156, 190  
 genetic alteration, 107  
 genetic factors, 2, 165  
 genetic mutations, 16  
 genetics, 19, 54, 164  
 genomic instability, 214  
 genotype, 7, 26  
 gland, 135, 154  
 glass transition temperature, 120, 129  
 glial cells, 7, 221  
 Glioblastoma, 192  
 glioma, 110  
 glucocorticoids, 174  
 glucose, 37, 52, 74, 86, 160, 195, 196, 197, 202, 223  
 glucoside, 72, 78  
 glutamate, 198, 208, 218, 223, 224  
 glutamine, vii, xiii, 189, 190, 191, 195, 196, 197, 198, 200, 201, 202, 203, 204, 207, 208, 209, 210, 211, 212, 213, 215, 216, 218, 219, 220, 221, 222, 223, 224, 225  
 glutathione, x, xiii, 62, 66, 74, 77, 88, 189, 190, 191, 195, 196, 197, 198, 199, 200, 201, 203, 204, 216, 218, 219, 222, 224  
 glutathione peroxidase (GSH-Px), x, 62, 74, 195  
 glycine, 219  
 glycogen, 64, 88, 195, 202, 224  
 glycosaminoglycans, 136  
 glycoside, xii, 131, 137, 139, 160  
 gonads, 194  
 green fluorescence protein (GFP), x, 96, 98, 99, 105, 106, 107, 108, 111  
 growth, 56, 96, 101, 103, 110, 111, 122, 132, 136, 154, 158, 195, 197, 201, 202, 203, 214  
 growth factor, 96, 101, 103, 110, 111, 195, 196, 202, 203  
 growth hormone, 214  
 Guangdong, 89, 91, 92  
 Guangzhou, 61, 89, 90, 91, 92



guar gum, v, vii, xi, 115, 116, 117, 119,  
120, 121, 122, 123, 124, 126, 127,  
128

## H

hair, 132, 136  
hairless, 139, 147  
half-life, 184  
hallucinations, 14  
head and neck cancer, 111  
healing, 136, 146, 178, 180, 181, 182,  
184, 186, 203  
health, xiv, 52, 86, 146, 152, 157, 163,  
165, 166, 169, 170, 196, 199, 208,  
214, 223  
health effects, 163  
health status, xiv, 163, 208  
heart disease, viii, 31, 33, 34, 35, 36, 54,  
87, 153, 157, 158, 167  
heart failure, 42  
heat shock protein, 55, 96, 99, 100, 197,  
204, 218, 225  
heat shock protein 90 kDa (HSP90), 96,  
99, 100  
hematologist, 182  
hematopoietic stem cells, 176  
hemoglobin, 62, 190  
hemorrhage, 62, 65, 81  
hepatic fibrosis, 45  
hepatic injury, 59  
hepatitis, 56  
hepatocellular carcinoma, 56  
hepatocytes, 41, 67, 78, 84, 93, 219  
hepatotoxicity, 39, 44, 85  
herpes simplex, 97  
high density lipoprotein, 54  
hip replacement, 214, 220  
hippocampus, 3  
histone, 76  
HIV, 50, 155, 166  
HLA, 214  
homeostasis, 6, 9, 47, 67, 81, 86, 88, 202  
homocysteine, 15

hormone, xi, xii, 52, 131, 132, 133, 136,  
140, 144, 145, 146, 151, 152, 153,  
154, 156, 157, 158, 159, 163, 164,  
166, 167, 168, 169, 214  
hormone levels, 169  
hospitalization, 219  
hTERT, 96, 97, 98, 99, 100, 102, 105,  
106, 107, 108, 109, 110  
HTRA2, viii, 2, 8, 9, 11, 16, 25  
human, 13, 17, 18, 27, 29, 39, 42, 44, 50,  
52, 54, 84, 96, 97, 99, 100, 101, 102,  
105, 106, 107, 109, 110, 111, 112,  
132, 133, 134, 135, 136, 138, 144,  
146, 147, 148, 163, 164, 183, 204,  
209, 217, 224  
human body, 144, 183  
human brain, 17  
human estrogen receptor, 134  
human health, 146  
human organisms, 133  
human remains, 163  
human skin, 147, 148  
human telomerase reverse transcriptase  
(hTERT), 96, 97, 98, 99, 100, 102,  
105, 106, 107, 108, 109, 110  
human telomerase RNA component  
(hTR), 96, 99, 100  
hydrogen peroxide, 11, 201, 203  
hydrolysis, 71, 143, 217, 221  
hydrophilicity, 121  
hydroxyl, 72, 132, 133, 190, 200  
hydroxyl groups, 133  
hygiene, 176, 177, 180, 182  
hypercalcemia, 174  
hypercholesterolemia, 38, 39, 40, 47, 51,  
53, 54  
hyperglycemia, 51, 204  
Hyperlipidemia, v, vii, viii, ix, 31, 32,  
33, 34, 35, 38, 41, 46, 48, 50, 59  
hyperplasia, 137, 147, 163  
hypertension, 47, 80, 155  
hypertrophy, 42, 57, 209, 215, 224  
hypotension, 17, 76, 80  
hypothesis, 9, 15, 37, 78, 210  
hypovolemia, 65

hypoxia, 62, 64, 65, 71, 80, 81, 83, 202

# I

iatrogenic, 156

identification, 13, 142, 149, 169

identity, viii, 32

idiopathic, 10, 20, 23, 156

IFN, 22

IL-13, 225

IL-8, 195, 224

illumination, 111

immune function, xiii, 207, 218, 221, 225

immune response, ix, 7, 32, 41, 203, 208, 216, 221

immune system, 3, 9, 49, 176, 208, 222, 223

immunity, 41, 50, 202

immunofluorescence, 71

immunomodulator, 184

improvements, 213

*in vitro*, vii, xii, 6, 12, 14, 27, 43, 45, 59, 132, 138, 217

*in vivo*, vii, ix, xii, 6, 7, 14, 27, 32, 36, 43, 45, 49, 58, 59, 68, 72, 98, 130, 132, 139, 147

incidence, 38, 167, 168, 175

indirect effect, 74

individuals, 5, 9, 11, 13, 36, 37, 42, 47, 52, 117, 162, 212, 214, 221

inducer, 42

induction, ix, x, 32, 46, 76, 84, 95, 97, 98, 101, 103, 104, 108

industrialized countries, 33

infants, 145, 191, 201

infarction, 33, 35, 49, 67, 155, 159

infection, x, 44, 95, 97, 101, 102, 105, 106, 108, 134, 155, 176, 179, 180, 182, 223

infertility, 156, 168

inflammasome, 40, 55

inflammation, 41, 43, 44, 45, 49, 54, 59, 77, 88, 175, 178, 218, 219, 224

inflammatory mediators, 208

ingestion, 51, 199, 209, 221, 222, 223

ingredients, xi, 131, 137

inheritance, 14, 23

inhibition, xiii, 14, 37, 54, 58, 68, 70, 78, 79, 82, 88, 104, 112, 143, 147, 190, 195, 201, 202, 208, 211, 218, 219

inhibitor, 11, 39, 40, 45, 50, 53, 68, 75, 76

initiation, 35, 185

injections, 38

injury(ies), 40, 41, 44, 45, 55, 57, 59, 66, 67, 70, 73, 75, 76, 79, 80, 82, 85, 87, 88, 89, 90, 91, 92, 93, 136, 195, 196, 198, 202

innate immune response, 41

innate immunity, 41, 50

inositol, 67

insulin, 37, 41, 51, 52

insulin resistance, 51

insulin sensitivity, 41

integrity, 65, 66, 124, 126, 138, 174, 196, 203

intelligence, 155

intensive care unit, 62

interface, 127

intermolecular interactions, 119

internalization, 97, 219

intervention, 49, 159, 174, 181, 195, 204

intestine, xiii, 36, 39, 67, 77, 78, 82, 89, 91, 92, 93, 117, 191, 197, 208, 217, 218

intoxication, 195

intravenously, 176, 180, 183, 184

ionizing radiation, 112

ions, xi, 66, 116, 119, 125, 128

iron, 36, 72, 78, 84, 147

irradiation, 105, 106, 149

ischemia, 66, 67, 70, 73, 75, 76, 80, 81, 82, 85

ischemia-reperfusion injury, 73, 76, 85

isoflavone, xii, 131, 140, 141, 142, 143, 145, 148, 149, 161

isoflavonoids, 133, 141

isolation, 149, 169

isomers, 134

**J**

Japan, 8, 95, 105, 109, 159, 169  
 Jordan, 204  
 juveniles, 201, 202

**K**

K<sup>+</sup>, x, 61, 68, 73, 76  
 kaempferol, 133  
 keratinocyte, 137, 138, 147  
 keratinocytes, 136, 139  
 kidney, 43, 68, 76, 83, 88, 90, 160, 177,  
 214  
 kinase activity, 12, 14, 25, 147  
 kinetics, xi, 45, 116, 124

**L**

labeling, 111  
 lactic acid, 66  
 large intestine, 117  
 larynx, 199  
 lead, 3, 4, 9, 10, 11, 12, 15, 34, 42, 63,  
 64, 118, 120, 178, 190, 200, 203  
 lesions, ix, 6, 32, 41, 47, 169  
 leucine, viii, 1, 28  
 leukemia, 76, 96, 101, 103, 191, 201  
 lignans, xii, 131, 133, 160  
 lipemia, ix, 32, 35, 44, 46, 49, 50  
 lipid metabolism, 14, 41, 46, 49  
 lipid peroxidation, 15, 77, 191  
 lipid peroxides, 72, 147  
 lipids, ix, 32, 34, 35, 40, 41, 44, 47, 52,  
 136, 155  
 lipoproteins, 34, 36, 39, 44, 49, 55, 56,  
 58  
 liposomes, 44, 139, 141  
 liquid chromatography, 139, 142  
 liver, ix, xiii, 32, 36, 37, 39, 40, 41, 43,  
 44, 45, 46, 47, 50, 52, 55, 56, 57, 58,  
 59, 76, 82, 87, 98, 106, 107, 159, 177,  
 191, 203, 208, 217  
 liver cells, 40, 41, 44, 45, 46, 50

liver damage, 41, 44, 217  
 liver disease, ix, 32, 33, 40, 47, 55, 159  
 liver failure, xiii, 208  
 liver function tests, 159  
 localization, 4, 10, 13, 14, 27, 86, 196  
 locus, 3, 8, 11, 13, 24, 28  
 logical reasoning, 157  
 long-term memory, 157  
 low fat diet, 51  
 low risk, 8  
 low-density lipoprotein (LDL), viii, ix,  
 31, 32, 33, 34, 35, 36, 37, 38, 39, 43,  
 44, 46, 50, 51, 53, 58, 157  
 LRRK2, viii, 1, 2, 7, 8, 9, 12, 14, 16, 22,  
 25, 26, 28, 29  
 luciferase, 72  
 luminescence, 72  
 lung cancer, 112, 113, 199, 202  
 Luo, 55, 87, 109, 145, 217, 222  
 luteinizing hormone, 156  
 lymph node, 98, 101, 106, 110, 111  
 lymphocytes, 196, 209, 223  
 lysine, 5, 20, 76, 216  
 lysis, x, 96, 99, 101, 102, 107, 109

**M**

macromolecular entanglements, 124  
 macrophages, ix, 32, 36, 40, 41, 42, 44,  
 45, 46, 47, 50, 55, 57, 58, 59, 208,  
 223  
 magnetic resonance imaging, 42  
 majority, 137, 156, 180  
 malignancy, 56  
 malignant mesothelioma, 110  
 malignant tumors, 191  
 malnutrition, 176, 199, 200, 204, 224  
 mammalian brain, 28  
 mammalian cells, 216  
 management, 52, 53, 109, 170, 186, 187,  
 200, 204  
 mandible, 179  
 manganese, 66, 87  
 manipulation, 54  
 mannans, ix, 32, 34, 38

- marrow, 199
- mass, xi, 19, 70, 116, 128, 139, 143,  
149, 174, 211, 217
- mass spectrometry, 139, 143, 149
- materials, xi, 115, 117, 120, 123, 125
- matrix, xi, 12, 54, 58, 63, 64, 66, 69, 71,  
82, 115, 116, 117, 118, 119, 120, 121,  
122, 123, 124, 126, 127, 128, 129,  
130
- matrix metalloproteinase, 54, 58
- matrixes, 70, 118, 119, 120
- maxilla, 176, 192
- maxillofacial area, 176
- measles, 97
- measurements, xiii, 68, 138, 140, 167,  
207
- mechanical properties, 145
- media, xi, 116, 118, 119, 120, 122, 123,  
124, 125, 127, 128
- medical, 62, 82, 156, 159, 166, 174, 183,  
184, 185, 186
- medical history, 183, 184, 185
- medication, 183, 185
- medicine, vii, ix, 32, 40, 42, 47, 83, 84,  
112, 164, 167, 170
- melatonin, 74, 77, 85, 88
- mellitus, 52, 62, 176, 177
- membranes, 69, 70, 75, 138, 174, 180
- memory, 155, 157, 214, 219
- menopause, xii, 132, 135, 140, 141, 144,  
145, 151, 152, 153, 154, 155, 156,  
157, 158, 159, 163, 164, 165, 166,  
169, 171
- menstrual cycles, 152, 154
- mesothelioma, 110
- messenger RNA, 38
- meta-analysis, 48, 161, 169, 170, 171
- Metabolic, 51, 81, 197
- metabolic pathways, xiii, 207, 208
- metabolism, vii, x, xiii, 14, 15, 39, 40,  
41, 46, 49, 54, 59, 61, 63, 66, 73, 74,  
76, 144, 148, 163, 166, 190, 195, 196,  
197, 200, 201, 202, 203, 207, 208,  
213, 219, 220, 223, 226
- metabolites, 63, 70, 87, 139, 190, 200
- metabolized, 3
- metalloproteinase, 54
- metastasis, 101, 107, 110, 111
- methanol, 143
- methodology, 226
- methylene blue, xi, 115, 117, 118, 119,  
120, 122, 125, 126
- Mexico, 189, 191, 194
- mice, ix, 21, 32, 35, 41, 44, 45, 46, 49,  
50, 54, 55, 56, 58, 59, 76, 87, 88, 106,  
203, 223
- microbiota, 217, 225
- microcirculation, 81, 89
- microemulsion, 149
- microorganisms, 180
- microRNA, 101, 103, 111, 112
- microscope, 70, 105
- microscopy, 43, 226
- Ministry of Education, 109
- mitochondria, viii, x, 2, 4, 9, 10, 11, 12,  
23, 61, 63, 66, 67, 68, 69, 70, 71, 72,  
73, 75, 76, 77, 78, 79, 80, 81, 83, 84,  
85, 86, 138, 197, 200, 217
- mitochondrial damage, x, 61, 63, 66, 68,  
72, 77, 83, 88, 92
- mitochondrial dysfunction (MD), x, 10,  
62, 63, 66, 68, 69, 70, 71, 72, 73, 75,  
76, 77, 78, 79, 80, 82, 83, 85, 86, 88,  
173, 196, 214
- mitochondrial inner membrane (MIM),  
x, 62, 63, 64, 71
- mitochondrial membrane potential  
( $\Delta\Psi_m$ ), x, 9, 62, 63, 71
- mitochondrial outside membrane  
(MOM), x, 62, 63
- mitochondrial permeability transition  
pore (mPTP), x, 61, 62, 63, 64, 66,  
68, 69, 70, 71, 72, 74, 75, 76, 78, 79,  
81, 82, 84, 88
- mitogens, 12, 196
- MMPs, 45, 49, 55, 58
- models, vii, xiii, 13, 14, 17, 35, 42, 75,  
101, 105, 107, 118, 119, 189, 195,  
203, 217
- modifications, 3, 4, 10, 21

moisture, 136, 140  
 moisture content, 136, 140  
 molecular mass, 70  
 molecular medicine, 112  
 molecular pathology, 80  
 molecular weight, 117  
 molecules, 70, 77, 81, 117, 119, 126, 128  
 MOM, x, 62, 63, 64  
 monoclonal antibody, 39, 44, 53  
 monomers, 3, 71  
 morbidity, viii, 31, 177, 201  
 morphology, 12, 70  
 mortality, viii, 31, 33, 35, 39, 40, 48, 54, 86, 201  
 Moses, 20  
 Mre11-Rad50-NBS1 (MRN), 96, 101, 104, 112  
 MRI, 186  
 mRNA, 13, 38, 136  
 mtDNA, viii, 2  
 mucosa, 217  
 mucous membrane, 174, 180  
 multiple myeloma, 174  
 muscle contraction, 67  
 muscle strength, 222  
 musculoskeletal, 175  
 mutant, 12, 18, 25, 27  
 mutations, 3, 4, 5, 6, 7, 10, 11, 12, 13, 14, 16, 18, 19, 20, 21, 22, 23, 24, 25, 26, 28, 29, 40, 77, 107, 108, 156  
 myalgia, 162  
 myeloid cell leukemia 1 (MCL1), 96, 101, 103, 112  
 myeloid cells, 90  
 myocardial infarction, 33, 35, 49, 155, 159

## N

Na<sup>+</sup>, 119  
 N-acetylcysteine (NAC), x, 62, 74  
 NaCl, 117, 119, 122, 123, 125  
 nanosystems, 141  
 nanotechnology, 147, 149

National Academy of Sciences, 19, 20, 21, 24, 145, 223  
 natural compound, 36, 163  
 neck cancer, 111  
 necrosis, 44, 80, 175, 180, 187  
 negative consequences, 195  
 negative effects, xiii, 173  
 neocortex, 3  
 neoplasm, 203  
 nerve, 3, 26  
 nervous system, vii, xiii, 1, 2, 3, 208  
 Netherlands, 11  
 neurodegeneration, 6, 13, 17, 26  
 neurodegenerative disorders, viii, 2, 9, 190  
 neuroimaging, 5, 20  
 neuroinflammation, 55  
 neuronal cells, 9, 190  
 neurons, viii, 2, 3, 4, 6, 7, 12, 13, 15, 17, 24, 73, 78  
 neurophysiology, 226  
 neurotoxicity, 12, 219  
 neurotransmitter, 6, 12, 13, 14, 27, 28  
 neutral, xiii, 124, 207  
 neutrophils, 208, 216, 217, 223  
 New England, 19, 164, 167  
 niacin, 219  
 nicotinic acid, 34  
 nigrostriatal, 2  
 nitric oxide, 18, 77, 204  
 nitrogen, 177, 214, 216  
 nitroxide, 75  
 nodes, 106  
 non-classical, 136  
 North Africa, 9, 18  
 North America, 152, 164, 166, 168  
 Nrf2, 23  
 nuclei, 3  
 nucleic acid, 29, 66  
 nucleotides, 99, 100  
 nucleus, 5, 136  
 nutrient, 71, 191, 196, 201, 202, 214, 222  
 nutrients, 65, 196, 199  
 nutrition, 191, 199, 201, 203, 204, 226

nutritional status, 42

## O

obesity, 52  
 oligomerization, 27  
 omega-3, 34  
 oophorectomy, 156  
 oral cavity, 174, 176, 177, 180, 182, 185  
 organ, x, 61, 62, 63, 65, 67, 75, 76, 79,  
     80, 83, 85, 98, 108, 137, 147, 156,  
     194, 199, 214  
 organelles, 10, 13, 63, 67  
 organic compounds, 132, 134  
 organism, 132, 133, 134, 135, 183, 199,  
     200  
 orthostatic hypotension, 17  
 osmotic pressure, 69, 70  
 osteonecrosis of the jaw, vii, xiii, 174,  
     175, 176, 179, 181, 184, 185, 186,  
     187  
 osteonecrosis prevention, 174  
 osteoporosis, 145, 153, 155, 157, 164,  
     174, 177, 185, 187  
 ototoxicity, 81  
 ovarian cancer, 112, 158, 163, 167  
 ovarian failure, 156, 166  
 ovaries, xii, 151, 152, 154, 156  
 overtraining, 224  
 overweight, 155  
 ovulation, 153, 154  
 oxidation, 15, 29, 66, 73, 190, 195, 221  
 oxidative damage, 6, 18, 77, 190  
 oxidative stress, viii, xiii, 2, 3, 5, 6, 10,  
     11, 15, 24, 66, 72, 74, 75, 78, 85, 88,  
     189, 190, 196, 197, 198, 199, 200,  
     202, 204, 210, 217  
 oxygen, x, 4, 16, 61, 62, 63, 65, 66, 71,  
     74, 78, 79, 138, 143, 190, 196, 216  
 oxygen consumption, 65

## P

p53, 68, 76, 78, 79, 83, 86, 87, 89, 91,  
     93  
 paclitaxel, 101  
 pain, 154, 162, 174, 175, 179  
 palpitations, 155  
 pancreas, 107  
 paranoia, 14  
 parasites, 198  
 parenchymal cell, 43  
 Parkin, v, viii, 1, 2, 3, 4, 5, 6, 9, 10, 16,  
     18, 19, 20, 22, 23, 25  
 parkinsonism, 4, 10, 14, 18, 19, 21, 23,  
     24, 27, 28, 29  
 participants, 48, 212, 213, 215  
 pathogenesis, vii, viii, 1, 2, 10, 14, 15,  
     16, 19, 25, 33, 41, 42, 46, 63, 77, 79  
 pathogens, 9, 22  
 pathology, 2, 8, 10, 17, 40, 42, 43, 44,  
     80, 165, 168  
 pathophysiological, 46  
 pathophysiology, 55, 180  
 pathways, vii, xiii, 2, 17, 20, 25, 26, 41,  
     45, 57, 68, 79, 80, 81, 82, 93, 103,  
     158, 190, 195, 196, 198, 200, 202,  
     207, 208, 211, 213, 217, 218, 219  
 PCR, 107, 136  
 penetrance, 23, 26  
 peptide, 180, 209, 210, 212, 214, 215,  
     221  
 performance measurement, xiii, 207  
 perfusion, 65  
 peripheral blood, 84, 113, 217, 218, 225  
 peripheral blood mononuclear cell, 217,  
     218, 225  
 peripheral nervous system, 3  
 peritoneal cavity, 98  
 permeability, x, 7, 61, 62, 63, 64, 74, 81,  
     82, 83, 84, 86, 88, 211, 217, 218, 225  
 permeation, xi, 116, 148  
 peroxidation, 15, 77, 138, 191  
 peroxide, 11, 201, 203  
 peroxyinitrite, 75  
 pH, xi, 116, 117, 125, 128, 223

- phagocyte, 43, 50  
phagocytosis, 7, 43  
pharmaceutical, 129, 184  
pharmacokinetics, 184  
pharmacology, 81, 86, 88  
pharmacotherapy, 16, 185  
phenotype, 7, 8, 10, 21, 23  
phenyl-tert-butyl nitrate (PBN), x, 62, 74, 75  
phosphorylation, 4, 5, 6, 63, 64, 67, 71, 73, 77, 104  
physical activity, 208, 214, 215, 216, 219, 221  
physiology, 146, 154, 214  
pilot study, 113  
PINK1, viii, 2, 4, 8, 9, 10, 11, 16, 22, 23, 25  
pituitary gland, 154  
placebo, 35, 37, 140, 147, 157, 161, 170  
plant sterols, 36, 37, 51, 52  
plants, ix, 32, 34, 132, 133, 134, 140, 160  
plasma levels, 196, 225  
plasma membrane, 67  
point mutation, 4, 5, 7, 20  
Poland, 1, 131, 151  
polarization, 46  
poloxamer 407, ix, 32, 35, 41, 46, 49, 56  
polydatin (PD), vii, viii, x, 1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, 15, 16, 29, 62, 69, 70, 72, 74, 78, 79, 90  
polymer, xi, 115, 116, 118, 120, 124, 125, 127, 128, 129  
polymeric materials, xi, 115  
polymers, 117, 124, 126  
polymorphisms, 7, 8, 10  
polyp, 154, 159, 164  
polyphenols, 133, 216  
polysaccharides, v, vii, viii, ix, 31, 32, 34, 35, 36, 38, 47, 117, 129  
polyunsaturated fatty acids, 138, 191, 201  
population, vii, ix, 1, 7, 9, 10, 11, 12, 32, 44, 46, 47, 48, 138, 169, 191  
potassium, 75, 82, 83  
potential benefits, 213  
prediction models, 35  
prefrontal cortex, 29  
pregnancy, 47, 154  
preparation, 117, 139, 141, 142  
preterm infants, 191, 201  
prevention, xiii, 11, 33, 34, 35, 37, 38, 48, 49, 73, 145, 149, 164, 167, 170, 174, 181, 185, 190, 196, 208, 210, 214, 215  
primary tumor, 101, 106  
probe, 71, 84, 140  
processing pathways, 57  
producers, 62, 132  
progenitor cell, 6, 195, 203  
progesterone, 154, 156, 157, 164  
progestins, 157  
prognosis, 73, 174, 183, 186, 194, 196  
pro-inflammatory, 46, 211  
proliferation, x, xiii, 63, 95, 99, 100, 108, 137, 138, 195, 203, 207, 208  
proline, 190, 216  
promoter, 7, 97, 98, 99, 100, 102, 105, 106, 108, 109, 110, 113  
prophylactic, 182, 183  
prostate carcinoma, 174  
proteasome, 3, 9, 11, 18, 20  
protection, xiii, 63, 75, 79, 80, 82, 89, 90, 91, 92, 93, 208  
protective factors, 4  
protective role, ix, 6, 32, 198, 204  
protein kinases, 67  
protein oxidation, 29  
protein structure, 26  
protein synthesis, xiii, 189, 214, 220  
proteinase, 39, 50  
proteins, vii, viii, x, 1, 2, 3, 4, 5, 10, 11, 13, 14, 15, 26, 62, 64, 66, 68, 69, 70, 73, 76, 77, 80, 134, 136, 198, 221  
proteoglycans, 136  
proteolysis, ix, 32, 50  
psychological distress, 165  
psychology, 155  
psychosocial factors, 165  
PTEN, viii, 2, 4

**Q**

quality of life, xii, 170, 173, 174, 185  
 quercetin, 133, 216

**R**

radiation, 102, 104, 105, 112, 113, 133, 134, 208  
 radiation therapy, 113  
 radiation treatment, 208  
 radicals, xii, 66, 72, 78, 131, 138, 147, 199  
 radiosensitization, 112  
 radiotherapy, xi, 96, 98, 101, 108, 176, 185, 190, 197  
 RBC, 107  
 reaction time, 210, 211, 212, 223  
 reactions, 67, 72, 158  
 reactive oxygen, x, 4, 16, 61, 62, 63, 66, 74, 79, 138, 143, 190, 196, 216  
 reactive oxygen species (ROS), x, 4, 9, 10, 12, 15, 16, 61, 62, 63, 66, 70, 72, 74, 75, 77, 79, 138, 143, 190, 195, 196, 197, 209, 216, 218  
 receptor, 5, 36, 39, 40, 49, 50, 76, 77, 90, 96, 97, 101, 103, 110, 111, 133, 134, 135, 136, 145, 146, 147, 160, 184  
 recognition, 200  
 recommendations, 167, 181, 183  
 recovery, xiii, 13, 14, 199, 200, 205, 208, 209, 212, 213, 215, 216, 222  
 red blood cells, 107  
 rehabilitation, 182, 200  
 rehydration, 216  
 relaxation, xi, 116, 118, 119, 120, 123, 127, 128  
 repair, viii, 2, 102, 104, 112, 190  
 replication, x, 96, 97, 98, 99, 100, 102, 105, 106, 108, 109, 110, 111, 112, 113  
 reproduction, 165, 166  
 reproductive organs, 156

requirement, viii, 2, 65, 199  
 RES, 89, 90, 91  
 residues, 5, 76, 140, 160  
 resistance, 9, 11, 34, 51, 198, 199, 202, 204, 209, 212, 215  
 respiration, 19, 68, 71  
 response, 3, 5, 7, 9, 10, 11, 12, 16, 22, 29, 35, 37, 42, 49, 54, 57, 65, 68, 77, 79, 86, 88, 104, 134, 135, 138, 154, 159, 190, 195, 196, 200, 201, 202, 203, 214, 216, 219  
 resveratrol (RSV), x, 62, 69, 70, 72, 74, 77, 78, 79, 87, 88, 134, 135, 145  
 reticulum, 39, 67  
 reverse transcriptase, 96, 97, 100, 102, 106, 109  
 rhabdomyolysis, 34  
 rheumatologist, 182  
 risk, viii, 7, 8, 10, 12, 13, 14, 16, 23, 31, 33, 34, 35, 36, 37, 38, 40, 48, 52, 54, 107, 109, 143, 152, 153, 155, 156, 157, 159, 163, 164, 165, 167, 168, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 200, 219  
 risk assessment, 35  
 risk factors, viii, 31, 35, 48, 165, 167, 176, 183, 186  
 risk management, 109  
 risk profile, 176  
 risks, 145, 159, 174, 180, 185  
 RNA, 38, 96, 99, 100  
 rubbery state, 123  
 ruthenium, 73, 74, 82

**S**

salts, 147  
 scavengers, 66, 74, 139  
 secretion, 36, 39, 45, 136, 154, 219, 225  
 selective estrogen receptor modulator, 133, 145, 148  
 senescence, x, 42, 57, 87, 95, 99, 100, 214  
 sensing, 214



- sensitivity, 10, 11, 41, 75, 107, 198  
sepsis, 75, 78, 80, 81, 83, 84, 85, 86, 88, 90, 217, 219, 220, 223  
septic shock, 75, 83  
sequencing, 107, 108  
serine, 11, 12, 53, 190, 201  
serum, ix, 32, 35, 36, 41, 44, 46, 49, 50, 51, 52, 55, 56, 59, 180, 195  
sex, xi, 81, 131, 132, 135, 152, 154, 156, 157, 158, 164  
sex differences, 164  
sex hormones, 135, 152, 156, 157, 158  
sex steroid, 156  
sexual activity, 155  
shock, vii, ix, 55, 61, 62, 63, 65, 66, 68, 69, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 88, 89, 90, 91, 92, 93, 96, 99, 100, 197, 204, 211, 217, 218, 219, 225  
shock therapy, x, 61  
side effects, 15, 34, 35, 42, 75, 116, 143, 159, 162, 185, 196  
signal transduction, 13  
signaling pathway, 103  
signs, xii, 5, 44, 59, 70, 151, 178  
skeletal muscle, 35, 44, 59, 86, 215, 224  
skin, xii, 3, 131, 132, 134, 135, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 154, 157, 162, 167, 171  
small intestine, 67, 77, 78, 82, 89, 91, 92, 93  
smoking, 155, 156, 166, 176, 177  
smooth muscle cells, x, 42, 57, 62, 69, 72, 76, 78, 80, 82, 83, 86  
sodium, 119, 121  
soft tissue sarcomas, 111  
solid tumors, 104, 112, 187  
solubility, 4, 6, 119, 121, 123, 124, 128, 140, 148  
solution, 75, 85, 119, 138, 143, 212  
solvents, 142  
somatic cell, 101, 105  
sorption, xi, 116  
soybeans, 159  
species, x, 4, 16, 38, 61, 62, 63, 66, 74, 77, 79, 138, 143, 180, 190, 196, 198, 216  
specific surface, 97  
spectroscopy, 45  
spinal cord, 3  
spleen, ix, 32, 57  
Sprague-Dawley rats, 217  
stability, 4, 72, 117, 148  
stabilization, 6, 75, 78  
starvation, 196  
state, 6, 16, 19, 43, 64, 65, 66, 70, 118, 123, 163, 168, 182, 183, 185, 190  
state of shock, 65  
statin, vii, viii, ix, 31, 32, 33, 34, 35, 38, 39, 41, 47, 48  
Statins, v, viii, 31, 34, 35, 48  
statistics, 47, 159, 191  
stem cells, 86, 176, 195  
steroids, 177  
sterols, 36, 37, 51, 52  
stimulation, 11, 36, 43, 49, 56, 67, 196  
stomach, 107  
storage, ix, 15, 28, 33, 41, 42, 47  
stress, viii, xiii, 2, 3, 4, 5, 6, 9, 10, 11, 15, 24, 33, 66, 68, 72, 74, 75, 78, 85, 87, 88, 133, 134, 155, 189, 190, 191, 196, 197, 198, 199, 200, 202, 204, 208, 210, 213, 214, 215, 216, 217, 221, 222, 224  
stress response, 68, 210, 214, 216  
striatum, 14, 88  
stroke, 35, 47, 88, 158, 159, 195, 203  
structure, viii, 2, 3, 5, 6, 7, 26, 64, 78, 123, 124, 133, 134, 135, 161  
subcutaneous injection, 38  
substrate, x, xiii, 4, 5, 61, 73, 74, 138, 189, 196, 218  
superoxide dismutase (SOD), x, 62, 66, 74, 79, 87, 138, 191  
supervisor, 226  
supplementation, vii, xii, xiii, 37, 38, 52, 140, 151, 159, 161, 191, 195, 196, 197, 201, 202, 203, 204, 207, 208, 209, 210, 211, 212, 213, 214, 215,

216, 217, 218, 220, 221, 222, 224, 225  
 suppression, ix, xi, xiii, 32, 50, 58, 68, 96, 108, 175, 208, 209, 213, 216  
 surgical intervention, 156, 181  
 survival, 6, 45, 75, 83, 85, 87, 104, 112, 191, 195, 202, 216  
 suture, 183  
 swelling, vii, xi, 63, 69, 70, 115, 116, 118, 119, 120, 122, 123, 124, 125, 126, 127, 128, 178, 179  
 swelling behavior, 116, 128  
 swelling kinetics, 124  
 symptomatic treatment, 165  
 symptoms, vii, viii, xii, xiii, 3, 7, 8, 10, 11, 12, 13, 14, 31, 45, 152, 153, 154, 155, 157, 158, 159, 161, 162, 165, 166, 169, 170, 173, 175, 179, 183  
 synapse, 28  
 synaptic plasticity, 6  
 synaptic transmission, 6, 14, 15  
 synaptic vesicles, viii, 2, 12, 13, 16  
 synchronization, xi, 116, 127  
 syndrome, 33, 35, 41, 46, 51, 80, 83, 156, 175, 204, 214  
 synergistic effect, 112, 143  
 synthesis, viii, xiii, 2, 6, 37, 38, 40, 52, 63, 68, 70, 71, 77, 137, 138, 140, 143, 189, 197, 204, 214, 216, 217, 219, 220, 224  
 synthesizing activity, 52

## T

T cell, 217  
 target, x, 5, 9, 34, 41, 42, 54, 66, 73, 74, 76, 77, 79, 83, 84, 95, 97, 99, 108, 190, 195  
 teeth, 175, 178, 186  
 telangiectasia, 112, 203  
 telomerase, vii, x, 95, 96, 97, 98, 99, 100, 102, 105, 106, 108, 109, 110, 111, 112, 113  
 telomerase-associated protein 1 (TEP1), 96, 99, 100

telomere, 95, 99, 100, 214  
 telomere shortening, 100  
 testosterone, 157, 158, 168  
 TGF, 136, 195, 196, 222  
 therapeutic approaches, 73  
 therapeutic effects, 141, 198  
 therapeutic interventions, 198  
 therapeutic targets, 46, 76, 77, 79, 89, 91, 92  
 therapy, vii, viii, x, xii, 3, 12, 15, 18, 29, 32, 33, 35, 37, 38, 41, 53, 61, 62, 73, 96, 98, 101, 105, 108, 109, 110, 113, 132, 140, 144, 145, 146, 152, 157, 158, 159, 160, 162, 163, 164, 166, 167, 168, 169, 170, 173, 174, 175, 176, 177, 181, 182, 183, 184, 185, 186, 187, 195, 200  
 tissue, 15, 42, 45, 62, 66, 67, 68, 75, 80, 86, 111, 135, 148, 158, 175, 181, 183, 184, 190  
 TMEM230, viii, 2, 13, 26  
 TNF, 43, 44, 217, 224  
 TNF-alpha, 43, 224  
 TNF- $\alpha$ , 217, 224  
 tobacco, 155, 156, 166  
 tobacco smoking, 155, 166  
 tooth, 178, 179, 186  
 total cholesterol, 33, 37, 38, 52  
 toxicity, 6, 7, 11, 13, 14, 15, 43, 84, 203, 204, 217  
 trafficking, 5, 12, 13, 14, 15, 28  
 training, 51, 165, 209, 212, 213, 215, 216, 221, 222  
 transcription, 46, 64, 101, 145, 190, 200  
 transcription factors, 46, 145, 190  
 transduction, xii, 13, 131, 147  
 transfection, 198  
 transferrin, x, 62, 73  
 transformation, 127, 138, 142  
 transforming growth factor, 195, 196, 202, 203  
 transfusion, 62  
 transition temperature, 120, 129  
 translocation, 12, 46, 76

transmission, 6, 12, 14, 15, 50, 70, 136, 138  
 transport, viii, x, xi, 2, 6, 12, 13, 14, 49, 62, 66, 73, 77, 116, 118, 120, 127, 128, 217  
 trauma, 196, 203, 214, 223  
 traumatic brain injury, 198  
 treatment, vii, viii, ix, x, xii, xiii, 2, 3, 5, 7, 9, 10, 13, 14, 15, 16, 17, 29, 31, 33, 34, 37, 38, 39, 40, 41, 43, 44, 45, 47, 48, 51, 61, 62, 63, 70, 73, 74, 78, 79, 80, 83, 84, 85, 89, 91, 92, 96, 97, 99, 105, 108, 109, 110, 138, 145, 146, 148, 152, 157, 159, 161, 164, 165, 173, 174, 175, 177, 180, 182, 183, 184, 185, 186, 187, 194, 195, 196, 201, 204, 215  
 tremor, 2, 9, 11, 12, 13, 27  
 trial, 49, 51, 104, 105, 147, 161, 168, 175, 191, 204, 213, 225  
 tricarboxylic acid, 73, 208, 218  
 tricarboxylic acid cycle, 73, 208  
 triglycerides, ix, 32, 39, 40, 46, 142  
 triphenylphosphonium (TPP<sup>+</sup>), x, 62, 75  
 tropism, 98, 110  
 trypsin, 195  
 tryptophan, 10  
 tumor, x, 49, 50, 56, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 168, 187, 191, 193, 198, 199  
 tumor cells, x, 95, 96, 97, 98, 99, 100, 101, 105, 107, 108, 109, 111, 112, 113, 199  
 tumor development, 49  
 turnover, viii, ix, 2, 33, 47, 57, 175  
 type 2 diabetes, 52  
 tyrosine, viii, xii, 2, 6, 45, 131, 138, 147  
 tyrosine hydroxylase, viii, 2

## U

ubiquitin, 3, 4, 5, 9, 10, 19, 20, 22  
 ubiquitin-proteasome system, 3  
 ultrasonography, 165

United Kingdom, 12  
 United States, 19, 20, 21, 24, 33, 104, 105, 108  
 urea, 214, 218  
 urinary tract, 154  
 urine, 183, 218  
 USA, 8, 31, 57, 157, 201  
 uterus, 154  
 UV irradiation, 149  
 UV radiation, 134

## V

vagina, 154, 158  
 vaginitis, 154  
 vagus nerve, 3  
 valuation, 214  
 variables, vii, 63, 72, 129  
 variations, 5, 12, 20  
 vascular wall, 33  
 vascularization, 136  
 vasomotor, 161, 169, 170  
 vegetables, 133  
 vesicle, 7, 12, 13, 14, 15, 19, 26, 27, 28  
 viral gene, 97  
 virotherapy, x, 95, 96, 97, 98, 99, 100, 101, 108, 109, 111, 112  
 virus infection, 97  
 virus particles (Vp), 96, 105  
 virus replication, x, 95, 97, 99, 100, 102, 106, 108  
 viruses, 97, 109  
 visualization, 98  
 vitamins, 88, 213  
 VLDL, 39  
 voltage-dependent anion channel (VDAC), x, 62, 63, 64  
 vulva, 154

## W

water, xi, 35, 36, 38, 52, 70, 115, 116, 117, 118, 119, 120, 121, 122, 123,

124, 125, 126, 127, 128, 129, 136,  
138, 140, 142, 210, 212

water diffusion, 119, 120, 123

well-being, 158, 168

withdrawal, 168, 190

worldwide, 7, 16, 26, 62

wound healing, 146, 180, 186, 203

**X**

xenografts, 110

**Y**

yeast, vii, viii, ix, 32, 34, 35, 38, 46

Yeast Polysaccharides, v, 31, 35

young women, 156

**Z**

zinc, 36