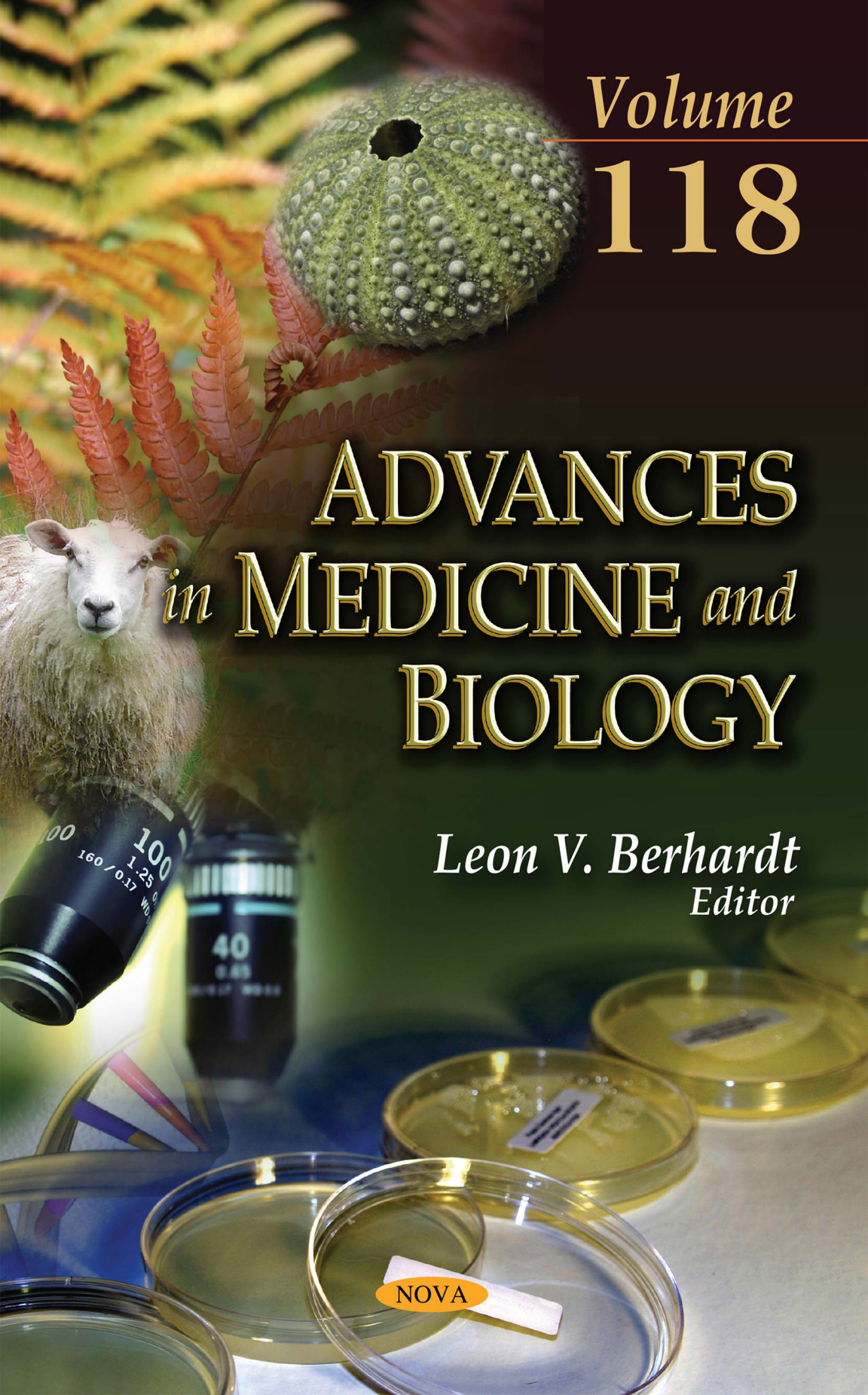
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ADVANCES
in MEDICINE and
BIOLOGY
Leon V. Berhardt
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ADVANCES IN MEDICINE AND BIOLOGY

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VOLUME 118

ADVANCES IN MEDICINE AND BIOLOGY

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ADVANCES IN MEDICINE AND BIOLOGY

ADVANCES IN MEDICINE AND BIOLOGY

VOLUME 118

LEON V. BERHARDT
EDITOR

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PREFACE

The chapters in this volume present the latest developments in medicine and biology. Chapters One researches recent topics in retinal ganglion cells. Chapter Two provides a review of acetylsalicylic acid resistance. Chapter Three analyzes the effect of chlorogenic acid (CGA) on myocardial diseases. Chapter Four focuses on the role of diet in the composition and activity of gut microbiota. Chapter Five examines data on the Maillard reaction as a cause of stochastic damage in *E. coli*, and subsequently, of *E. coli* aging. Chapter Six studies haploinsufficient tumor suppressor genes. Chapter Seven discusses animal models of respiratory distress in neonates. Chapter Eight presents professional training in the work-up of bleeding disorders in women. Chapter Nine investigates microbial dynamics characterization during the composting process of organic wastes.

Chapter 1 - Retinal photoreceptors (rods and cones) respond only to light falling on the retina from a specified direction. This allows them to detect the amount of light originating from a small point in viewing space. Having photoreceptors is a prerequisite for the spatial resolution necessary for any reasonable level of visual acuity. However, simpler organisms have also evolved photoreceptive mechanisms without a visual system. What then do these simpler organisms use light detection for, and is this evolutionarily ancient function still relevant for photoreception?

The transmission of visual information to visual centers via the optic nerve involves light perception by rods and cones, conversion to electrical signals, and generation of action potentials by retinal ganglion cells (RGCs). The photoreception by which light emitted from a point in viewing space is perceived and an image of the external world is captured, and this is known as image-forming vision. Although the rods and cones play an important role, the

RGCs probably plays only a minor role in this photoreception. Over 90% of the total number of RGCs include *parasol* RGCs that respond to low contrast stimulation and *midget* RGCs that process images from red and green color information.

Recently, the existence of RGCs with specialized functions, such as perceiving object movement and direction (directional selective RGCs), light-dark contrast boundaries (local edge detector RGCs), and processing blue color information (blue-ON / yellow-OFF RGCs), has been reported. Moreover, these are classified not as a single type, but rather many types (at least 13 distinct types), of neurons based on their dendritic morphologies (spread, degree of branching, linearity), projection sites of the inner plexiform layer, and response to ON/OFF light.

Chapter 2 - Aspirin has been used for a hundred years to prevent platelets from forming thromboxane A2 in atherosclerotic events. Today, many researchers are investigating the possible mechanisms of aspirin resistance. The first survey on this topic was done by Mehta et al. in 1978; they gave 650 mg aspirin to 10 patients before catheterization. They reported that the platelet function remained normal during and after catheterization in three of these patients. Other surveys have investigated why some people are affected more and others less. Initially, there is failure in the formation of thromboxane A2; after treatment, failure is due to various additional factors. Patient compliance can play an important role in aspirin resistance. There is no standardized laboratory test for aspirin resistance. In treatment, it is important to know the underlying reason. An exact diagnosis and treatment are necessary with diseases other than those with thrombotic causes, such as infective endocarditis and vasculitis. Medications that can interact with aspirin have to be avoided, and the patient must give up smoking. The other significant point is the need for dose adjustment according to the individual patient.

Chapter 3 - It is well known that plant polyphenols inhibit the development of cardiovascular diseases. However, the effect of chlorogenic acid (CGA), one of the major polyphenol constituents of coffee beans, on the disease is yet to be elucidated. This article reviews the effect of CGA on myocardial diseases. Firstly, the authors reviewed the effects of CGA on human cardiovascular diseases. Several studies indicated that CGA might decrease the clinical risk of cardiovascular diseases via anti-hypertensive and anti-endothelial dysfunction. Next, the authors demonstrated that CGA could improve pathological remodeling through the suppression of myocardial cell infiltration and fibrosis in animal models. The authors' findings and previous

data suggest that the CGA treatment may have beneficial effects on the progression of myocardial diseases.

Chapter 4 - Gut microbiota represents an important part in food digestion, immunity, metabolism and gene expression as well. It consists of about 10^{14} bacterial cells represented by more than 400 species that can affect its host in many ways. Qualitative and quantitative composition of microbiota can be influenced by many factors such stress, age, antibiotic therapy or diet. The last mentioned factor is a study subject of many researchers nowadays. Many studies have pointed out that diet based on vegetables such vegan diet shift human gut microbiota to higher prevalence of beneficial bacteria and consequently to healthier lifestyle. In the authors' research the authors have studied qualitative and quantitative composition of cultivable bacterial genera in stool of 240 healthy volunteers aged between 20 and 60 differ in diet. More than half (136) belongs to vegetarians divided to semi vegetarians (pullo and pesco), lacto vegetarians, lacto-ovo vegetarians and vegans. No significant differences were found between meat-eaters and vegetarians. On the other hand, lacto-, vegans had lower number of almost all studied bacterial genera. The authors have also focused on presence of potential mutagenic compounds in stool. Percentage of stools positive on potential mutagenic compounds was almost same in both groups. However, obesity is also linked to shifts in microbial composition, obese and lean subjects were also studied. Obese subjects have only slightly increased staphylococci. Furthermore, the authors have observed more differences between underweight and lean subjects.

Chapter 5 - Recent studies have challenged the paradigm that bacteria do not age and are immortal. Stewart et al. (PLoS Biology, 2005, 3(2), e45) provided an intriguing evidence that during division *Escherichia coli* K-12 exhibits functional asymmetry, leaving behind a mother cell with delayed growth and survival rate and a younger daughter cell, empowered to successfully perpetuate the species over time. In view of this new finding, bacteria, and in particular *E. coli*, emerged as a promising model for exploration of basic mechanisms of aging. It is not yet clear to what extent pro- and eukaryotic cells age similarly but at least some features of aging, especially at the molecular level, should be common. Spontaneous chemical reactions including hydrolysis, oxidation and glycation (the Maillard reaction) are well known to deteriorate macromolecular structure and function. The Maillard reaction, yielding the so-called Advanced Glycation End Products (AGEs) on proteins, DNA and amino-lipids, has been long associated with diseases (diabetes, Alzheimer's and Parkinson's diseases) and aging in humans. The authors have demonstrated that despite the short life span of *E.*

coli of tens of minutes to hours, its chromosomal DNA accumulates AGEs under normal growth conditions (Mironova et al., Mol. Microbiol., 2005, 55(6), p1801). Such progressive modification of a key biological molecule provides an independent line of evidence for *E. coli* aging. This article reviews data on the Maillard reaction as a cause of stochastic damage in *E. coli*, and subsequently, of *E. coli* aging.

Chapter 6 - Haploinsufficiency of tumor suppressor genes (TSGs) indicates that the reduced levels of proteins in cells that lack one allele of the genomic locus results in the inability of the cell to execute normal cellular functions contributing to tumor development. Representative cases of haploinsufficient TSGs are $p27^{Kip1}$, *p53*, *DMP1*, *NF1*, and *PTEN*. Tumor development is significantly accelerated in both mice with homozygous and heterozygous gene deletion, with expression of the wild type allele in the latter. Newly characterized TSGs such as *AML1*, *EGR1*, *TGFβR1/2*, and *SMAD4* have also shown haploid insufficiency for tumor suppression. This phenotype has typically been demonstrated in gene knockout mouse models, but analyses of human samples have been conducted in some cases. Recent studies suggest collaboration of multiple haploinsufficient TSGs in 5q-, 7q-, and 8q- syndromes, which is called compound haploinsufficiency. Although *ARF* is a classical TSG, it also belongs to this category since $Arf^{+/-}$ accelerates tumor development when both alleles for *Ink4a* are inactivated. Haploid insufficiency of *Arf* was also reported in myeloid leukemogenesis in the presence of inv(16). In case of p53, $p53^{+/-}$ cells achieve only ~25% of p53 mRNA and protein levels as compared to those in wild type, which could explain the mechanism. $TGF\beta R1^{+/-}$ collaborates with $Apc^{Min+/-}$ in colorectal cancer development; $TGF\beta R2^{+/-}$ and $Smad4^{+/-}$ collaborates with *K-Ras* mutation in pancreatic ductal adenocarcinomagenesis, demonstrating the synergism of haploinsufficient TSGs and other oncogenic events. These TSGs can be targets for activation therapy in cancer since they retain a functional allele even in tumor cells.

Chapter 7 - A respiratory distress in neonates developed from various reasons is characterized by a reduced ability or an inability to supply gas exchange. This situation may originate from a prematurity and an insufficient synthesis of a pulmonary surfactant, i.e., respiratory distress syndrome (RDS) in preterm neonates. Another reason is represented by a secondary inactivation of the surfactant in the term neonates, e.g., from bacterial endotoxins in pneumonia or from aspiration of a meconium. To find out an appropriate treatment, a special *in vivo* testing in the laboratory conditions is necessary. The models of the mentioned clinical situations provide new information on

the pathophysiology of the disorders. Furthermore, they offer unique possibilities to test novel therapeutic approaches in the conditions very similar to the respective clinical syndromes.

Chapter 8 - More than 2 million women in the United States have an underlying bleeding disorder. The prevalence for bleeding disorders among women with menorrhagia is 20%. The most commonly reported symptoms among individuals with a diagnosis of von Willebrand disease (VWD) or any suspected bleeding disorder include epistatxis, gingival bleeding, easy bruising and menorrhagia. Therefore, patient history is very important to guide an initial workup. Additionally, many recommend that the initial evaluation include complete blood count (CBC), prothrombin time (PT), activated partial thromboplastin time (PTT), international normalized ratio (INR), platelet function assay (PFA-100), blood type, bleeding time and VWF assays. There are a variety of treatments that exist for von Willebrand factor (VWF). The National Heart, Lung, and Blood (NHLBI) of the National Institutes of Health issued a guideline regarding bleeding disorders. This chapter describes the highlights of the NHLBI guidelines. In addition, the authors include information on the training of obstetrics-gynecology residents and Women's Health Advance Practice Nurses in the evaluation of bleeding disorders in women, and characterize the experience more fully and assess the evolving state of training in the evaluation of menorrhagia and bleeding disorders.

Chapter 9 - Composting process is a natural pathway to convert organic matter into humic substances end product. The objective of this study is focused mainly on the characterization of the microflora, which plays a key role as decomposers during the composting process and its interaction with physico-chemical parameters. Mesophilic microflora constitutes the pioneer component, while thermophilic microflora contributes significantly to the quality of the compost especially during the thermophilic stage. Indigenous microflora community changes during composting of activated sewage sludge and date palm waste, and food waste and garden clippings. The thermophilic microflora evolution during composting is linked to ascending temperature (over than 50°C). The mesophilic microflora is found to be more abundant than the thermophilic bacteria throughout the co-composting process. Total microflora followed the temperature profile variation. The values of pH, T°C and C/N ratio are typical values for composting process. The temperature rise and the abatement of C/N ratio during composting of sewage sludge/ date palm and household/green waste indicate the intense microbial activity. Thereafter, the decrease in biomass and temperature show the end of the composting process. Fungal microflora increases during the thermophilic stage

with a high correlation rate in sewage sludge-palm waste composting. The changes in microflora explain the organic matter evolution during composting. The dynamics of microbial community in different compost ecosystems varies with qualitative and quantitative changes in physico-chemical conditions of compost, which is linked to the quality of organic matter degradation.

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Chapter 1

RECENT TOPICS IN THE RETINAL GANGLION CELLS

Ken Asakawa*
Department of Orthoptics and Visual Science, Kitasato University, School of Allied Health Sciences, Japan

1. ROLE OF RODS, CONES AND RETINAL GANGLION CELLS

Retinal photoreceptors (rods and cones) respond only to light falling on the retina from a specified direction. This allows them to detect the amount of light originating from a small point in viewing space. Having photoreceptors is a prerequisite for the spatial resolution necessary for any reasonable level of visual acuity. However, simpler organisms have also evolved photoreceptive mechanisms without a visual system. What then do these simpler organisms use light detection for, and is this evolutionarily ancient function still relevant for photoreception?

The transmission of visual information to visual centers via the optic nerve involves light perception by rods and cones, conversion to electrical signals, and generation of action potentials by retinal ganglion cells (RGCs). The photoreception by which light emitted from a point in viewing space is

* Corresponding author: Ken Asakawa, 1-15-1 Kitasato, Sagamihara, Minami-ku, Kanagawa 252-0373, Japan. E-mail: asaken@kitasato-u.ac.jp

perceived and an image of the external world is captured, and this is known as image-forming vision. Although the rods and cones play an important role, the RGCs probably plays only a minor role in this photoreception. Over 90% of the total number of RGCs include *parasol* RGCs that respond to low contrast stimulation and *midget* RGCs that process images from red and green color information.

Recently, the existence of RGCs with specialized functions, such as perceiving object movement and direction (directional selective RGCs), light-dark contrast boundaries (local edge detector RGCs), and processing blue color information (blue-ON / yellow-OFF RGCs), has been reported. Moreover, these are classified not as a single type, but rather many types (at least 13 distinct types), of neurons based on their dendritic morphologies (spread, degree of branching, linearity), projection sites of the inner plexiform layer, and response to ON/OFF light. [1]

2. MELANOPSIN-CONTAINING RETINAL GANGLION CELLS

Retinal photoreceptor cells were regarded as the rods and cones for more than 100 years, but the fact the circadian rhythm is maintained even in mice and humans who have completely lost vision suggested the presence of other photoreceptors. Provencio et al. [2] isolated melanopsin, a photosensitive visual pigment, from dermal melanophores in *Xenopus laevis* and reported that melanopsin was present in RGCs of inner retina. These RGCs respond to depolarization regardless of visual information from conventional photoreceptors of outer retina, and they are known as intrinsically photosensitive RGCs or melanopsin-containing RGCs (mRGCs).

mRGCs account for only about 1% of the total number of RGCs, but have large cell bodies and an extensively branching dendritic structure with long dendrites. [3] Therefore, mRGCs make it possible to detect light over an extensive portion of the retina despite their relatively small number. Berson et al. [4] found that mRGCs have projections to the suprachiasmatic nucleus (SCN), the center of the internal clock, and reported these as novel photoreceptors involved in circadian rhythm. Some nerve terminals project to the SCN and play a role in regulation of the circadian rhythm and melatonin secretion. [5] In particular, they also project to the olivary pretectal nucleus (OPN) and Edinger-Westphal nucleus (EW) in the midbrain and play a role in the pupil light reflex. [6, 7] These photoreceptions by mRGCs are called non-image-forming vision, and based on wavelength characteristics that lead to

pupil constriction in mice [7] and rabbits [8] with complete retinal photoreceptors loss or dysfunction, sensitivity is highest for 460-480 nm blue light and become depolarized in response to light stimulus after a long latency time.

Recently, they can be classified into five subtypes (M1-M5) based on differences in their dendrite formation and projection sites, [9] but differences in their function, significance, and expressed genes are not well understood. In our previous experiment [10], this about 470-nm blue light is the same wavelength as the color of the daytime blue sky **(**Figure 1**)**. This fact indicates that the presence of a mechanism for perceiving blue light dates back to ancient organisms, and that non-image-forming vision is related to natural light.

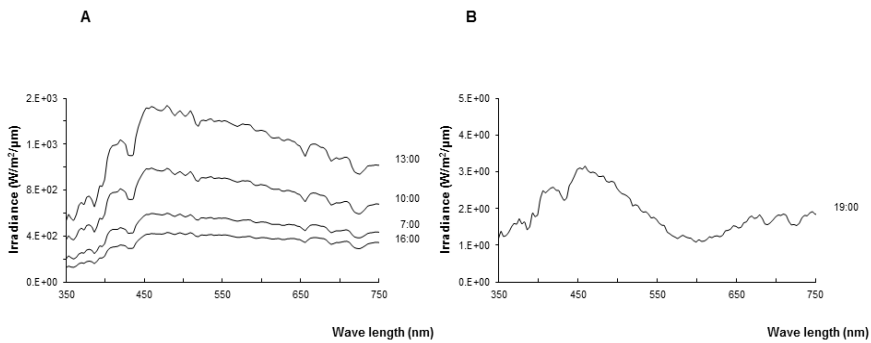

Figure 1. Wavelength components of natural light. Natural light contained wavelengths of blue light with peak intensities at 470 nm (A: from 7:00 to 16:00, B: 19:00 only). (Modified and reproduced with permission from reference 10.)

3. Contribution of MRGCs to the Pupil Light Response

The blue light stimulus activates mostly S cones and rods, in addition to any possible activation of mRGCs. The effect on that should be considered; accordingly, it is still not firmly established whether the obtained response was caused by the mRGCs. Thus, we performed the animal experiment in rabbits with degeneration of rods and cones. [8, 11] This experiment was conducted according to the ARVO Statement, and was approved by the Animal Experiment Ethics Committee of Kitasato University. We conducted the functional evaluation by recording the changes in the pupil response to red (635 nm) and blue (470 nm) light stimulus and the amplitude

of the electroretinography (ERG). Morphologically, rod and cone distribution was examined using light microscopy and electron microscopy. Immunofluorescence staining for the identification of mRGCs was confirmed by injecting a specific antibody.

Although the flat ERG, pupil constriction with a long latency time was still induced during exposure to blue light (Figure 2). Of note, some RGCs had large cell bodies with long branching dendritic structures, and the mRGCs could be identified even in a rabbit (Figure 3). Consequently, these findings indicate that the colored-light pupil response can be used to predict the state of outer retinal degeneration. Moreover, the existence of these residual mRGCs despite advanced degeneration of conventional retinal photoreceptors i.e., rods and cones indicates the fact that mRGCs are involved in synchronization of the circadian rhythm and are classical photoreceptors in the sleep-wake cycle.

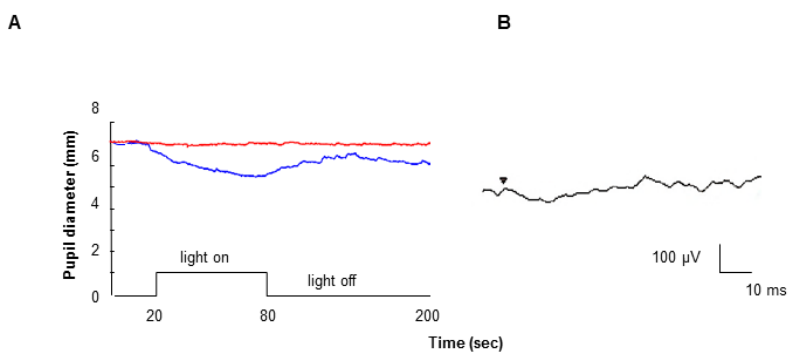

Figure 2. Typical pupillography and electroretinography in rabbit with outer retinal degeneration.

A. Pupil constriction with long latency time is induced with blue light stimulus, but not with red light stimulus. B. Amplitudes of a-and b-waves were almost non-recordable. Small arrowhead shows the start point of light stimulus.

(Modified and reproduced with permission from reference 8.)

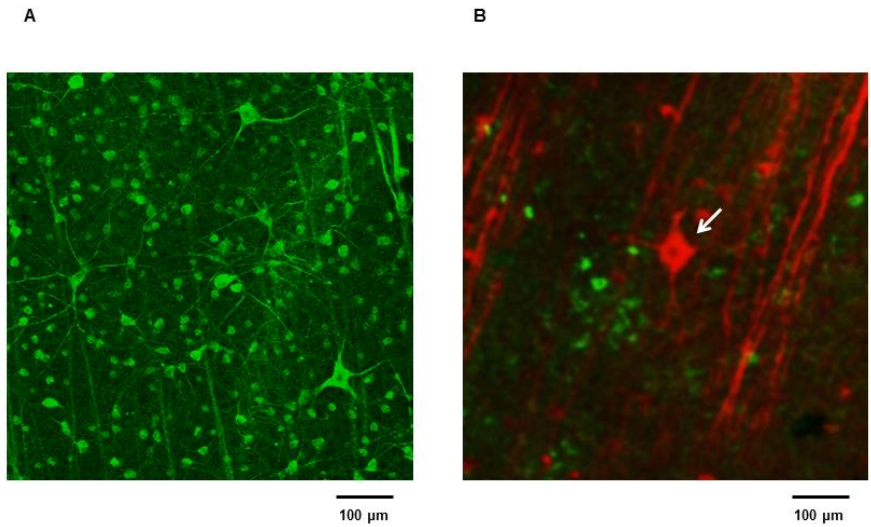

Figure 3. Various types of rabbit RGCs.

A. Both large cells and small cells, and also some large cells with long branching dendrites are observed. B. mRGC can be identified even in the rabbit (white arrow).

4. Future Outlook of mRGCs

Reports of circadian rhythm changes with the ambient light-dark cycle even in a mouse model of retinal degeneration with complete loss of rods and cones has prompted further basic research on mRGCs. [12] On the other hand, in mice with specific loss of mRGCs, projections to the SCN are lost, the circadian rhythm is not synchronized, and the pupil light reflex is also completely lost. Interestingly, visual perception ability is similar to wild-type mice, and thus various studies have reported that they also contribute to visual perception. [13] In monkeys, the projection to the lateral geniculate nucleus (LGN), a major pathway of visual information, have been found. [3] Mice with rods and cones dysfunction are reported to have a spatial resolution of <0.16 cycles/degree. [14] Moreover, humans in whom rods and cones function has been lost can still detect light at a wavelength of 480 nm. [15] If indeed mRGCs can contribute to a visual perception at a specific light wavelength, these can be developed for alternative vision in patients with retinal photoreceptors dysfunction.

ACKNOWLEDGMENT

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REFERENCES

[1] Sanes JR, Masland RH. The types of retinal ganglion cells: current status and implications for neuronal classification. *Annu. Rev. Neurosci.* 38: 221-246, 2015.

[2] Provencio I, Rodriguez IR, et al. A novel human opsin in the inner retina. *J. Neurosci.* 20: 600-605, 2000.

[3] Dacey DM, Liao HW, et al. Melanopsin-expressing ganglion cells in primate retina signal color and irradiance and project to the LGN. *Nature* 433: 749-754, 2005.

[4] Berson DM. Strange vision: ganglion cells as circadian photoreceptors. *Trends Neurosci.* 26: 314-320, 2003.

[5] Hattar S, Liao HW, et al. Melanopsin containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity. *Science* 295: 1065-1070, 2002.

[6] Lucas RJ, Douglas RH, et al. Characterization of an ocular photopigment capable of driving pupillary constriction in mice. *Nat. Neurosci.* 4: 621-626, 2001.

[7] Gooley JJ, Lu J, et al. A broad role for melanopsin in nonvisual photoreception. *J. Neurosci.* 23: 7093-7106, 2003.

[8] Ishikawa H. Pupil and melanopsin photoreception. Nippon Ganka Gakkai Zasshi 117: 246-268, 2013 [in Japanese].

[9] Chen SK, Badea TC, et al. Photoentrainment and pupillary light reflex are mediated by distinct populations of ipRGCs. *Nature* 476: 92-95, 2011.

[10] Asakawa K, Ishikawa H. Why do melanopsin-containing retinal ganglion cells have the greatest sensitivity to blue light? *Acta Ophthalmol.* 93: 308-309, 2015.

[11] Asakawa K, Ishikawa H. Functional and morphological study of retinal photoreceptor cell degeneration in transgenic rabbits with a Pro347Leu rhodopsin mutation. *Jpn. J. Ophthalmol.* 59: 353-363, 2015.

[12] Ebihara S, Tsuji K. Entrainment of the circadian activity rhythm to the light cycle: effective light intensity for a Zeitgeber in the retinal degenerate C3H mouse and the normal C57BL mouse. *Physiol. Behav.* 24: 523-527, 1980.

[13] Hatori M, Le H, et al. Inducible ablation of melanopsin-expressing retinal ganglion cells reveals their central role in non-image forming visual responses. *PLoS One* 3: e2451, 2008.

[14] Ecker JL, Dumitrescu ON, et al. Melanopsin-expressing retinal ganglion-cell photoreceptors: cellular diversity and role in pattern vision. *Neuron* 67: 49-60, 2010.

[15] Zaidi FH, Hull JT, et al. Short-wavelength light sensitivity of circadian, pupillary, and visual awareness in humans lacking an outer retina. *Curr. Biol.* 17: 2122-2128, 2007.

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Chapter 2

A REVIEW OF ACETYLSALICYLIC ACID RESISTANCE

Bulut Demirel, MD*
Department of Emergency Medicine, Ankara Atatürk Education and Research Hospital, Ankara, Turkey

ABSTRACT

Aspirin has been used for a hundred years to prevent platelets from forming thromboxane A2 in atherosclerotic events. Today, many researchers are investigating the possible mechanisms of aspirin resistance. The first survey on this topic was done by Mehta et al. in 1978; they gave 650 mg aspirin to 10 patients before catheterization. They reported that the platelet function remained normal during and after catheterization in three of these patients. Other surveys have investigated why some people are affected more and others less. Initially, there is failure in the formation of thromboxane A2; after treatment, failure is due to various additional factors. Patient compliance can play an important role in aspirin resistance. There is no standardized laboratory test for aspirin resistance. In treatment, it is important to know the underlying reason. An exact diagnosis and treatment are necessary with diseases other than those with thrombotic causes, such as infective endocarditis and vasculitis. Medications that can interact with aspirin have to be

* Corresponding Author: Bulut Demirel, MD, Ankara Education and Research Hospital, Ankara, Turkey.

avoided, and the patient must give up smoking. The other significant point is the need for dose adjustment according to the individual patient.

1. INTRODUCTION

Acetylsalicylic acid (ASA) has been used for more than a hundred years to inhibit the formation of thromboxane A2 by platelets during atherosclerotic events. However, ASA resistance is reported with increased frequency. One of the first studies on this topic was performed by Mehta et al. [1] in 1978. They detected ASA resistance in three-tenths of a group of patients who took 650 mg ASA prior to catheterization. A similar study was performed in 1993 with 181 patients who were given 500 mg ASA three times a day. Their platelet functions were measured, and 60 of the patients were detected as being ASA resistant. At a two-month follow up, 40% of the resistant patients and 4.4% of the non-resistant patients had been faced with a severe vascular event [2]. An increasing number of studies on ASA resistance are being conducted. In these studies, the prevalence of ASA resistance is shown to be between 5.5% and 61% [3-8].

2. PHYSIOLOGY OF PLATELET ADHESION, ACTIVATION AND AGGREGATION

When a normal vessel's intima layer and subendothelial collagen are injured, the von Willebrand factor is released into the circulation. The platelets in circulation bind with the subendothelial collagen by their glycoprotein Ia/IIa and Ib/V/IX receptors; this platelet adhesion causes an activation which results in a change in the platelet shapes [9-11]. At the same time, due to certain chemical reactions, the bound calcium in the platelets changes into a free type. The free ion calcium causes additional changes in the platelets. The first change is to the structural change of the glycoprotein IIb/IIIa that is present on the surface, resulting in fibrinogens in the circulation binding to the proteins. The second change in the platelet activation is the binding to surrounding platelets through the provision of molecules such as ADP. The third change is the formation of arachidonic acid due to the increased effect of phospholipase A2 [9-11].

The arachidonic acid that is present in the platelets is transformed into thromboxane A2 due to the effects of COX-1 and thromboxane synthase. Thromboxane A2 increases the fibrinogen receptors on the surface of the platelet so when it is released into the circulation it causes activation of the platelets by binding with their surface receptors [12]. Thromboxane A2 is a vasoconstrictor and also has a synergistic effect with proteins such as ADP, fibrinogen and factor V, which are released by the activated platelets.

2.1. Thromboxane A_2 Production

The production rate of thromboxane A2 can be measured from its metabolites such as thromboxane B2 in plasma and blood or 11-dehydrothromboxane in urine. The presence of thromboxane B2 in blood mainly depends on COX-1 [13]. However, the measurement of thromboxane B2 is difficult: production of thromboxane A2 other than by platelets can influence the results. The other thromboxane A2 production mechanisms are the macrocytes and macrophages inside the atherosclerotic plaques. In some instances, megakaryocytes and endothelial cells can also produce thromboxane A2 [14-18].

2.2. The Effect of ASA

ASA irreversibly inhibits the COX-1 enzyme by acetylation. In this way, the production of thromboxane A2 by the platelets is decreased [19-21]. This COX-1 inhibition is fast, is effective even in low doses and is irreversible. After a single 325 mg dose of ASA, the COX-1 activity by the reproduced platelets returns to normal by 10% each day. Thromboxane B2 (an enduring metabolite of thromboxane A2) decreases by 95% with the use of 0.45 mg/kg ASA for five days [21, 22]. It is thought that ASA has an additional antithrombotic effect, apart from COX-1, but there is no proven mechanism.

3. ASA RESISTANCE

ASA resistance can be divided into clinical and laboratory resistance according to the means of diagnosis [23].

3.1. Clinical ASA Resistance

The clinical ASA resistance can be explained as the failure of ASA to prevent the patient from experiencing atherosclerotic events. The diagnosis is determined after an atherothrombotic event in a patient who is using ASA in therapeutic doses. But this diagnostic method is a retrospective way of diagnosis and so is of limited benefit. Hence, this condition is called a failure of ASA treatment rather than ASA resistance. The factors thought to cause ASA resistance are summarized in Table 1.

Table 1. The possible causes of ASA resistance

A decrease in the bioavailability of ASA
• Inappropriate ASA usage (weak compliance with medication)
• Inappropriate ASA dose
• Decreased absorption or increased metabolism of ASA
Weak binding to COX-1
• Concomitant with use of some NSAID drugs
Other sources of thromboxane production that cannot be blocked by ASA
• Thromboxane production in macrophages, monocytes and endothelial cells due to COX-2
Other paths of platelet activation
• Increased hypersensitivity to collagen and ADP
• Platelet activation from the paths that cannot be blocked by ASA (collagen, ADP, epinephrine)
Increased platelet cycle
• Increased platelet formation in bone marrow due to stress, resulting in inadequate inhibition of COX-1 by daily ASA intake due to faster platelet cycle
Genetic polymorphisms
• Polymorphism in enzymes of COX-1, COX-2, thromboxane A synthase and other arachidonic acid metabolism enzymes
• Polymorphism in receptors of glycoprotein Ia/IIa, Ib/V/IX and IIb/IIIa and receptors of collagen and von Willebrand factors
Loss of antiplatelet effect due to long use of ASA
• Tachyphlaxis
Non-atherothrombotic vascular reasons
• Cardiac embolism (fibrin thrombus, tumor, prosthesis)
• Arteritis

3.2. Laboratory ASA Resistance

ASA resistance can also be diagnosed by laboratory examination measuring thromboxane A2 resistance or platelet function dependent on thromboxane [24-26]. The advantages and disadvantages of these laboratory tests are summarized in Table 2.

Table 2. Laboratory tests for measuring ASA resistance

Thromboxane Production	Advantages	Disadvantages
Blood thromboxane B2	It depends on the target of ASA treatment, COX-1	It may be non-specific to platelets The experience of the operator plays an important role
Urinary 11-dehydrothromboxane B2	It depends on the target of ASA treatment, COX-1 It has relationship with clinical events	Non-specific to platelets Sensitivity is unknown Repeatability is unknown Not studied much
Platelet function depending on thromboxane		
Light and optic aggregation	Conventional gold standard Widely used technique It has relationship with clinical events	Non-specific to platelets Sensitivity is not clarified Repeatability is limited Requires intensive workload It depends on the experience of the operator
Self-resistance aggregation	The preparation phase is easy	Non-specific to platelets Sensitivity is unknown It depends on the experience of the operator
PFA*-100	Easy Fast Semiautomatic It has relationship with clinical events	It depends on the level of von Willebrand factor and hematocrit Non-specific to platelets Sensitivity is unknown
Ultegra RFPA**	Easy Fast Semiautomatic It has relationship with clinical events	Sensitivity is unknown Specificity unknown

* PFA: platelet function analyzer.

** RPFA: Rapid platelet function analyzer.

3.3. The Causes of ASA Resistance

The possible mechanisms of aspirin resistance are expressed in Table 1. The classification of ASA resistance is explained by Weber et al. but the clinical classification and usefulness are not yet proven [8].

3.3.1. Weak Compliance with Medication

Weak compliance is an important factor that influences ASA resistance. The studies show that weak compliance is present in 40% of ASA users [27, 28]. In the case of weak compliance, clinical and laboratory resistance are inevitable.

3.3.2. Dose of ASA

Laboratory studies have shown that ASA can inhibit COX-1 effectively even with doses as low as 30 mg/day [19-22, 29]. Another study reported that with doses of ASA varying between 75 and 1300 mg/day, the effect of ASA does not change, but side effects such as gastrointestinal hemorrhage increase [12, 30, 31]. If the ASA dose is increased from 100 mg/day to 300 mg/day, the laboratory results change, but the clinical outcome does not [29, 32-35].

3.3.3. Intestinal Absorption and Metabolism of ASA

ASA absorption starts in the stomach because of its weak acidic features, thus soluble ASA reaches its peak effect within 30 to 40 minutes, but enteric coated aspirin reaches its peak effect in 3 to 4 hours. ASA is hydrolyzed into salicylic acid, which is its inactive form due to the effect of numerous mucosal esterases [36, 37]. The use of proton pump inhibitors (PPI) can increase the amount of mucosal esterase, thus enteral active ASA absorption can be decreased. The decreased effect of low doses of ASA can also be explained by this mechanism.

3.3.4. Metabolism in Portal Circulation

Absorbed ASA is hydrolyzed into inactive salicylic acid in the erythrocytes and liver cells, thus the effect of aspirin is seen in the pre-systemic circulation, and it has no effect in the systemic circulation. The plasma half-life is fifteen minutes.

3.3.5. The Binding of ASA to COX-1

Many of the non-steroidal anti-inflammatory drugs (NSAIDs) decrease the effect of ASA by blocking the binding of ASA to COX-1 [38, 39]. This

mechanism is explained in an observational study investigating the mortality of patients using ASA [40]. However, this mechanism has not yet been clarified.

3.3.6. Sources of Thromboxane A2 Other Than from Platelets

Arachidonic acid turns into thromboxane A2 in the monocytes and macrophages by thromboxane synthase with COX-2 [14-16, 18]. The effect of COX-2 is particularly increased in patients with atherosclerosis. The other mechanism is irrelevant with COX being produced by F2-isoprostans synthesized by free oxygen radicals produced by the lipid peroxidation of arachidonic acid [13, 41, 42]. Isoprostans increase with smoking, and hyperlipidemia also plays a role in ASA resistance [42, 43]. So, this circumstance could be seen as a relationship between vascular events, increased platelet factors and increased risk factors.

3.3.7. Other Pathways of Platelet Activation

The platelets can only be activated by thromboxane A2. The collagen (Ia/IIa) bound to glycoprotein receptors, the von Willebrand factor (Ib/V/IX), ADP, thrombin, epinephrine and the stretch stress on platelets also play a role in this activation [9-11]. In-vitro studies show the response of platelets to ADP and collagen in cases of ASA resistance; this response becomes more powerful in the presence of F2-isoprostans [13, 41, 44, 45].

3.3.8. Increased Platelet Cycle

In cases of coronary artery bypass graft (CABG) surgery, infection and inflammation from the platelet cycle are accelerated, so daily use of ASA, which has a short half-life, is incapable of inhibiting the newly produced COX-1 enzyme due to the accelerated platelet cycle [46].

3.3.9. Genetic Polymorphism

When a single-nucleotide polymorphism affects COX-1, COX-2 and other platelet genes, the antiplatelet effect of ASA changes [47-51]. Although there are hundreds of single-nucleotide polymorphisms, the effects are not yet known [52, 53]. One-third of the genes causing ASA resistance are detected by laboratory studies [49]. The gene most investigated in this context is P2Y1, which is the gene for the ADP receptor; in a case where there is any mutation on this gene, the ADP signal ability changes and the response of the thrombocytes to ASA can be decreased [50, 54, 55].

3.3.10. Tachyphylaxis

Tachyphylaxis is the loss of the effect of ASA due to long term use. In the beginning, ASA will regularly affect COX-1, but after months or years it loses this ability [56, 57]. The exact mechanism is not known, but we know that the atherosclerotic plaques are enlarged and that patient compliance decreases with time.

3.4. Diagnostic Methods for ASA Resistance

3.4.1. To Measure the Platelet Functions Dependent on Thromboxane

3.4.1.1. Light and Optic Transmission Aggregometer

The principle behind this technique is the increase of light passing through a suspension due to platelet aggregation under the influence of an agonist such as thromboxane A2, ADP or collagen. It is the gold standard method for measuring the antiplatelet effect of ASA; at the same time it is used to determine platelet functions. Arachidonic acid is more useful than the other agonists in measuring the effect of ASA because ADP and collagen have their effects on platelet aggregation over pathways that are less thromboxane production dependent.

3.4.1.2. Impedance Aggregometer

The principle here is to measure the aggregation response to platelet agonists by measuring the electrical impedance between two electrodes. The method is very similar to the light and optic transmission aggregometer, but the main advantage is that whole blood can be used rather than a platelet suspension, thereby making it is easier to use. Additionally, it can be used in thrombocytopenic patients.

3.4.1.3. Limitations of Light or Optic Transmission and Impedance Aggregometers

Results from both these methods can change according to age, gender, race and hematocrit level [13]. Furthermore, accuracy and repeatability are controversial. These are not standardized methods; the results can differ between laboratories.

3.4.1.4. Platelet Function Analyzer (PFA-100)

The principle behind this method is to measure bleeding time. An artificial reservoir is formed to represent a vessel. The material is put into this reservoir. The kit in the center includes collagen and ADP, or collagen and epinephrine. The kit moves as if in a vessel and is exposed to negative pressure to imitate the circulation. The artificial vessel is occluded in time and this time is recorded. The PFA-100 method changes according to the erythrocyte, platelet function, platelet amount and plasma von Willebrand factor levels [3]. However, some studies report that PFA-100 and light or optic aggregation do not reflect the clinical status of the patient [58].

3.4.1.5. Ultegra RPFA-ASA (Accumetrics, San Diego, CA, ABD)

The method can be applied at the bedside. Balls covered with fibrinogen are stimulated with arachidonic acid and agglutination is measured. If the ASA has achieved the desired antiplatelet effect, the fibrinogen coated balls do not undergo agglutination and light transmission does not increase. This result is called the ASA response unit.

The limitation of this test is as follows: after a single 325 mg dose of ASA, the test gives a result similar to the light and optic aggregation method as a result of the aggregation of platelets due to epinephrine [59, 60].

Another method used is the Adrenalin Dependent Platelet Aggregation. This is a sensitive method but it is not specific and its suitability for patients who use low doses of ASA is not known.

There are other methods used for measuring ASA resistance, but the studies are inadequate. The light or optic aggregation method is still the gold standard method, as the other methods have not yet been correlated [61]. This means that the laboratory diagnosis of ASA resistance changes according to the method used.

3.5. The Relationship between ASA and Clinical Status

The first clinical survey investigating ASA resistance was performed in 1993 and included 181 stroke survivor patients who used 500 mg of ASA three times a day. Their platelet functions were measured and 60 of the patients were defined as ASA resistant. After two years of follow up, 40% of the ASA resistant patients but only 4.4% of the other patients had been faced with a severe vascular event [2].

As a part of the HOPE Study (Heart Outcomes Prevention Evaluation) which was carried out in 2002, ASA treatment was applied at a dose of 75-325 mg/day to 976 patients with a high vascular risk and the 11-dehydrothromboxane B2 level was measured in their urine. After an average 4.5 years of follow up, there was a strong relationship between vascular events and 11-dehydrothromboxane B2 level. This correlation was not classified according to categories and also shows a linear raise in blood cholesterol levels [3].

3.6. Treatment for ASA Resistance

The main principle of treatment for ASA resistance is to determine the exact mechanism of the resistance. Other non-thrombotic causes such as infective endocarditis and vasculitis have to be diagnosed and treated regularly. At the same time, ASA does not prevent every ischemic event [62]. Medication that can interact with ASA has to be avoided and it is essential that patients give up smoking. Additionally, the dose adjustment has to be specific for the patient. However, even if all these factors are attended to, the treatment can still be unsuccessful [46].

In a case of ASA resistance, we are able to change the antiplatelet treatment, but resistance to other anti-platelet treatments are reported by some studies [63, 64]. This makes the antiplatelet treatment more complicated.

ASA resistance can change over time. The blood cholesterol level and blood pressure can both influence the action mechanism of ASA even when the absorption of ASA is normal. In time, the ASA resistance level can change, so ASA resistance has to be measured repeatedly in studies evaluating ASA resistance rather than relying on a single measurement [3-6, 8, 13, 23].

FURTHER STUDIES

We need to develop valid, specific, fast, cheap and repeatable methods for measuring platelet function. The patient population must be as large as possible and multicenter studies should be performed for more accurate results. The race, gender, age, co-morbid diseases, smoking habits, patient compliance, other medications and blood laboratory test results all have to be included as potential mediating factors in further studies. Other factors unrelated to platelets such as genetic polymorphism need to be evaluated.

Early detection of a mechanism of genetic resistance can potentially prevent severe vascular events.

In conclusion, ASA resistance has many complicated aspects that need to be clarified. It is to be hoped that new technologies and further research will provide us with more useful results.

REFERENCES

[1] Mehta J, Mehta P, Burger C, ve ark. Platelet aggregation studies in coronary artery disease. Past 4. Effect of aspirin. *Atherosclerosis*. Oct 1978;31(2):169-175.

[2] Grotemeyer K-H, Scharafinski H-W, Husstedt I-W. Two-year follow-up of aspirin responder and aspirin non responder. A pilot-study including 180 post-stroke patients. *Thrombosis research*. 1993;71(5):397-403.

[3] Gum PA, Kottke-Marchant K, Poggio ED, ve ark. Profile and prevalence of aspirin resistance in patients with cardiovascular disease. *The American journal of cardiology*. Aug 01 2001;88(3):230-235.

[4] Hankey GJ, Eikelboom JW. Aspirin resistance. *The Lancet*. 2006; 367(9510):606-617.

[5] Eikelboom JW, Hankey GJ. Failure of Aspirin to Prevent Atherothrombosis. *American Journal of Cardiovascular Drugs.* 2004; 4(1):57-67.

[6] Campbell C, Steinhubl S. Variability in response to aspirin: do we understand the clinical relevance? *Journal of Thrombosis and Haemostasis.* 2005;3(4):665-669.

[7] Sanderson S, Emery J, Baglin T, ve ark. Narrative review: aspirin resistance and its clinical implications. *Annals of Internal Medicine*. 2005;142(5):370-380.

[8] Weber A-A, Przytulski B, Schanz A, ve ark. Towards a definition of aspirin resistance: a typological approach. *Platelets*. 2002;13(1):37-40.

[9] McNicol A, Israels SJ. Platelets and anti-platelet therapy. *Journal of pharmacological sciences.* Dec 2003;93(4):381-396.

[10] Patrono C, Bachmann F, Baigent C, ve ark. Expert consensus document on the use of antiplatelet agents. The task force on the use of antiplatelet agents in patients with atherosclerotic cardiovascular disease of the European society of cardiology. *European heart journal*. Jan 2004; 25(2):166-181.

[11] Roth GJ, Calverley DC. Aspirin, platelets, and thrombosis: theory and practice. *Blood*. Feb 15 1994;83(4):885-898.
[12] Roth GJ, Majerus PW. The mechanism of the effect of aspirin on human platelets. I. Acetylation of a particulate fraction protein. *The Journal of clinical investigation*. Sep 1975;56(3):624-632.
[13] Maclouf J, Folco G, Patrono C. Eicosanoids and iso-eicosanoids: constitutive, inducible and transcellular biosynthesis in vascular disease. *Thrombosis and haemostasis*. Apr 1998;79(4):691-705.
[14] Karim S, Habib A, Levy-Toledano S, ve ark. Cyclooxygenase-1 and -2 of endothelial cells utilize exogenous or endogenous arachidonic acid for transcellular production of thromboxane. *The Journal of biological chemistry*. May 17 1996;271(20):12042-12048.
[15] Rocca B, Secchiero P, Ciabattoni G, ve ark. Cyclooxygenase-2 expression is induced during human megakaryopoiesis and characterizes newly formed platelets. *Proceedings of the National Academy of Sciences*. 2002;99(11):7634-7639.
[16] Weber AA, Zimmermann KC, Meyer-Kirchrath J, ve ark. Cyclooxygenase-2 in human platelets as a possible factor in aspirin resistance. *Lancet*. Mar 13 1999;353(9156):900.
[17] FitzGerald GA, Oates JA, Hawiger J, ve ark. Endogenous biosynthesis of prostacyclin and thromboxane and platelet function during chronic administration of aspirin in man. *Journal of Clinical Investigation*. 1983;71(3):676.
[18] Patrignani P, Sciulli MG, Manarini S, ve ark. COX-2 is not involved in thromboxane biosynthesis by activated human platelets. *Journal of physiology and pharmacology : an official journal of the Polish Physiological Society*. Dec 1999;50(4):661-667.
[19] Roth GJ, Stanford N, Majerus PW. Acetylation of prostaglandin synthase by aspirin. *Proceedings of the National Academy of Sciences of the United States of America*. Aug 1975;72(8):3073-3076.
[20] Burch JW, Stanford N, Majerus PW. Inhibition of platelet prostaglandin synthetase by oral aspirin. *The Journal of clinical investigation*. Feb 1978;61(2):314-319.
[21] Patrignani P, Filabozzi P, Patrono C. Selective cumulative inhibition of platelet thromboxane production by low-dose aspirin in healthy subjects. *The Journal of clinical investigation*. Jun 1982;69(6):1366-1372.
[22] Bhatt DL, Topol EJ. Scientific and therapeutic advances in antiplatelet therapy. *Nature reviews Drug discovery*. Jan 2003;2(1):15-28.

[23] Rand ML, Leung R, Packham MA. Platelet function assays. *Transfusion and apheresis science : official journal of the World Apheresis Association : official journal of the European Society for Haemapheresis*. Jun 2003;28(3):307-317.

[24] Harrison P. Progress in the assessment of platelet function. *Br J Haematol.* Dec 2000;111(3):733-744.

[25] Michelson AD. Platelet function testing in cardiovascular diseases. *Circulation*. Nov 9 2004;110(19):e489-493.

[26] Patrono C. Aspirin resistance: definition, mechanisms and clinical read-outs. *Journal of thrombosis and haemostasis : JTH*. Aug 2003;1(8):1710-1713.

[27] Tarjan J, Salamon A, Jager R, ve ark. [The rate of acetylsalicylic acid non-respondents among patients hospitalized for acute coronary disease, previously undergoing secondary salicylic acid prophylaxis]. *Orvosi hetilap.* 1999;140(42):2339-2343.

[28] Trialists'Collaboration A. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. *Bmj*. 2002;324(7329):71-86.

[29] Hart RG, Leonard AD, Talbert RL, ve ark. Aspirin dosage and thromboxane synthesis in patients with vascular disease. Pharmacotherapy: *The Journal of Human Pharmacology and Drug Therapy*. 2003;23(5):579-584.

[30] Serebruany VL, Steinhubl SR, Berger PB, ve ark. Analysis of risk of bleeding complications after different doses of aspirin in 192,036 patients enrolled in 31 randomized controlled trials. *The American journal of cardiology*. 2005;95(10):1218-1222.

[31] Roller R, Dorr A, Ulrich S, ve ark. Effect of aspirin treatment in patients with peripheral arterial disease monitored with the platelet function analyzer PFA-100. *Blood coagulation and fibrinolysis*. 2002;13(4):277-281.

[32] Cattaneo M. Aspirin and clopidogrel efficacy, safety, and the issue of drug resistance. *Arteriosclerosis, thrombosis, and vascular biology*. 2004;24(11):1980-1987.

[33] Dippel DW, van Kooten F, Leebeek FW, ve ark. What is the lowest dose of aspirin for maximum suppression of in vivo thromboxane production after a transient ischemic attack or ischemic stroke? *Cerebrovascular Diseases.* 2004;17(4):296-302.

[34] Cerletti C, Dell'Elba G, Manarini S, ve ark. Pharmacokinetic and pharmacodynamic differences between two low dosages of aspirin may affect therapeutic outcomes. *Clinical pharmacokinetics*. 2003;42(12): 1059-1070.

[35] Needs CJ, Brooks PM. Clinical pharmacokinetics of the salicylates. *Clinical pharmacokinetics*. 1985;10(2):164-177.

[36] Williams FM. Clinical significance of esterases in man. *Clin Pharmacokinet.* Sep-Oct 1985;10(5):392-403.

[37] Catella-Lawson F, Reilly MP, Kapoor SC, ve ark. Cyclooxygenase inhibitors and the antiplatelet effects of aspirin. *New England Journal of Medicine*. 2001;345(25):1809-1817.

[38] Capone ML, Sciulli MG, Tacconelli S, ve ark. Pharmacodynamic interaction of naproxen with low-dose aspirin in healthy subjects. *Journal of the American College of Cardiology*. Apr 19 2005;45(8): 1295-1301.

[39] MacDonald T, Wei L. Effect of ibuprofen on cardioprotective effect of aspirin. *The Lancet*. 2003;361(9357):573-574.

[40] Cipollone F, Ciabattoni G, Patrignani P, ve ark. Oxidant stress and aspirin-insensitive thromboxane biosynthesis in severe unstable angina. *Circulation*. 2000;102(9):1007-1013.

[41] Patrono C, Falco A, Davì G. Isoprostane formation and inhibition in atherothrombosis. *Current opinion in pharmacology*. 2005;5(2):198-203.

[42] Morrow JD, Frei B, Longmire AW, ve ark. Increase in circulating products of lipid peroxidation (F2-isoprostanes) in smokers—smoking as a cause of oxidative damage. *New England Journal of Medicine.* 1995;332(18):1198-1203.

[43] Macchi L, Christiaens L, Brabant S, ve ark. Resistance to aspirin in vitro is associated with increased platelet sensitivity to adenosine diphosphate. *Thrombosis research*. 2002;107(1):45-49.

[44] Kawasaki T, Ozeki Y, Igawa T, ve ark. Increased platelet sensitivity to collagen in individuals resistant to low-dose aspirin. *Stroke.* 2000;31 (3):591-595.

[45] Zimmermann N, Wenk A, Kim U, ve ark. Functional and biochemical evaluation of platelet aspirin resistance after coronary artery bypass surgery. *Circulation*. 2003;108(5):542-547.

[46] Gurbel PA, Bliden KP, Hiatt BL, ve ark. Clopidogrel for coronary stenting response variability, drug resistance, and the effect of pretreatment platelet reactivity. *Circulation*. 2003;107(23):2908-2913.

[47] Cipollone F, Toniato E, Martinotti S, ve ark. A polymorphism in the cyclooxygenase 2 gene as an inherited protective factor against myocardial infarction and stroke. *Jama.* 2004;291(18):2221-2228.

[48] Cambria-Kiely JA, Gandhi PJ. Aspirin resistance and genetic polymorphisms. *Journal of thrombosis and thrombolysis*. 2002;14(1): 51-58.

[49] Andrioli G, Minuz P, Solero P, ve ark. Defective platelet response to arachidonic acid and thromboxane A2 in subjects with PlA2 polymorphism of β3 subunit (glycoprotein IIIa). *Br J Haematol.* 2000;110(4):911-918.

[50] Michelson AD, Furman MI, Goldschmidt-Clermont P, ve ark. Platelet GP IIIa PlA polymorphisms display different sensitivities to agonists. *Circulation.* 2000;101(9):1013-1018.

[51] O'Donnell CJ, Larson MG, Feng D, ve ark. Genetic and Environmental Contributions to Platelet Aggregation The Framingham Heart Study. *Circulation*. 2001;103(25):3051-3056.

[52] Macchi L, Christiaens L, Brabant S, ve ark. Resistance in vitro to low-dose aspirin is associated with platelet PlA1 (GP IIIa) polymorphism but not with C807T (GP Ia/IIa) and C-5T Kozak (GP Ibα) polymorphisms. *Journal of the American College of Cardiology*. 2003;42(6):1115-1119.

[53] Jefferson BK, Foster JH, McCarthy JJ, ve ark. Aspirin resistance and a single gene. *The American journal of cardiology*. Mar 15 2005;95(6): 805-808.

[54] Szczeklik A, Undas A, Sanak M, ve ark. Relationship between bleeding time, aspirin and the PlA1/A2 polymorphism of platelet glycoprotein IIIa. *Br J Haematol*. 2000;110(4):965-967.

[55] Helgason CM, Bolin KM, Hoff JA, ve ark. Development of aspirin resistance in persons with previous ischemic stroke. *Stroke.* 1994;25(12): 2331-2336.

[56] Pulcinelli FM, Pignatelli P, Celestini A, ve ark. Inhibition of platelet aggregation by aspirin progressively decreases in long-term treated patients. *Journal of the American College of Cardiology*. 2004;43(6): 979-984.

[57] Warlow C, Sudlow C, Dennis M, ve ark. Stroke. *Lancet.* Oct 11 2003;362(9391):1211-1224.

[58] Malinin A, Spergling M, Muhlestein B, ve ark. Assessing aspirin responsiveness in subjects with multiple risk factors for vascular disease with a rapid platelet function analyzer. *Blood coagulation and fibrinolysis.* 2004;15(4):295-301.

[59] Harrison P, Segal H, Blasbery K, ve ark. Screening for Aspirin Responsiveness After Transient Ischemic Attack and Stroke Comparison of 2 Point-of-Care Platelet Function Tests With Optical Aggregometry. *Stroke*. 2005;36(5):1001-1005.

[60] Malinin AI, Atar D, Callahan KP, ve ark. Effect of a single dose aspirin on platelets in humans with multiple risk factors for coronary artery disease. *European journal of pharmacology*. 2003;462(1):139-143.

[61] Mueller MR, Salat A, Stangl P, ve ark. Variable platelet response to low-dose ASA and the risk of limb deterioration in patients submitted to peripheral arterial angioplasty. *Thrombosis and haemostasis*. 1997;78 (3):1003-1007.

[62] Van Gijn J. From randomised trials to rational practice. *Cerebrovascular Diseases*. 2005;19(2):69-76.

[63] Matetzky S, Shenkman B, Guetta V, ve ark. Clopidogrel resistance is associated with increased risk of recurrent atherothrombotic events in patients with acute myocardial infarction. *Circulation*. 2004;109(25): 3171-3175.

[64] Bhatt DL, Topol EJ, Committee CE. Clopidogrel added to aspirin versus aspirin alone in secondary prevention and high-risk primary prevention: rationale and design of the Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management, and Avoidance (CHARISMA) trial. *American heart journal*. 2004;148(2):263-268.

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Chapter 3

THE EFFECTS OF CHLOROGENIC ACIDS ON MYOCARDIAL DISEASES

Junichi Suzuki[1,*], MD, PhD
and Hirofumi Zempo[2], PhD
[1]Department of Advanced Clinical Science and Therapeutics, University of Tokyo, Tokyo, Japan
[2]Department of Cardiovascular Medicine, Tokyo Medical and Dental University, Tokyo, Japan

ABSTRACT

It is well known that plant polyphenols inhibit the development of cardiovascular diseases. However, the effect of chlorogenic acid (CGA), one of the major polyphenol constituents of coffee beans, on the disease is yet to be elucidated. This article reviews the effect of CGA on myocardial diseases. Firstly, we reviewed the effects of CGA on human cardiovascular diseases. Several studies indicated that CGA might decrease the clinical risk of cardiovascular diseases via anti-hypertensive and anti-endothelial dysfunction. Next, we demonstrated that CGA could improve pathological remodeling through the suppression of myocardial cell infiltration and fibrosis in animal models. Our findings and previous

[*] Correspondence Author Address: Department of Advanced Clinical Science and Therapeutics, University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-8655, Japan. Email, junichisuzuki-circ@umin.ac.jp.

data suggest that the CGA treatment may have beneficial effects on the progression of myocardial diseases.

Keywords: coffee extract, polyphenol, heart, inflammation

1. INTRODUCTION

Chlorogenic acid (CGA), a major polyphenol constituent of coffee beans, is a potent anti-oxidant and anti-inflammatory substance [1, 2]. These effects are induced by the suppression of inflammatory factors, including nuclear factor-kappa B (NF-κB) [3, 4]. Resent data has also indicated that CGA inhibits interleukin (IL)-1β and tumor necrosis factor (TNF)-α production in animals with rheumatoid arthritis [5]. However, the effect of CGA on myocardial disease is yet to be elucidated. Therefore, this article reviews the effect of CGA on myocardial disease.

2. THE EFFECTS OF CGA ON CARDIOVASCULAR DISEASES IN CLINICAL STUDIES

European studies showed that moderate coffee drinking reduced cardiovascular risk by 31% relative to no consumption. However, over consumption of coffee significantly increased the risk [6]. A similar J-curve was also observed in a Finnish study [7]. A study of over 40,000 post-menopausal US women showed the hazard ratio of death attributed to cardiovascular diseases was 0.76 for consumption of 1-3 cups/day, 0.81 for 4-5 cups/day, and 0.87 for ≥6 cups/day [8]. Thus, moderate consumption of coffee may inhibit inflammation and reduce the risk of cardiovascular and other inflammatory diseases.

Few clinical studies of CGA's influence on cardiovascular diseases have been performed. A study showed that the CGA significantly lowered systolic and diastolic blood pressure compared with the placebo group [9]. Normotensive subjects with reduced vasoreactivity were also administered with CGA. There was a significant decrease in plasma homocysteine compared with the baseline value for the CGA group [10]. These results indicate that CGA may decrease the risk of cardiovascular diseases.

3. THE EFFECTS OF CGA ON CARDIOVASCULAR DISEASES IN ANIMAL STUDIES

A study showed the effect of CGA on spontaneously hypertensive rats. A single ingestion of CGA reduced blood pressure in the rats, an effect that was blocked by administration of a nitric oxide synthase inhibitor. When spontaneously hypertensive rats were fed diets containing 0.5% CGA for 8 weeks, the development of hypertension was inhibited compared with the control diet group. Dietary CGA also reduced oxidative stress and improved nitric oxide bioavailability by inhibiting excessive production of reactive oxygen species in the vasculature, and led to the attenuation of endothelial dysfunction [11]. At a more relevant dose, CGA given to mice at 10 mg/kg activated calcineurin and enhanced macrophage functions in normal mice, a possible cardiac benefit [12].

4. CGA ON MYOCARDIAL INFARCTION

The mortality rate in patients with coronary arterial disease, including myocardial infarction (MI), is high [13]. Myocardial necrosis and ventricular remodeling after MI lead to arrhythmia, cardiac rupture and heart failure. Many studies showed that macrophage related inflammatory response increased myocardial necrosis [14, 15]. These inflammatory responses lead to an increase of fibroblasts and collagen synthesis [16-19]. To test the hypothesis that CGA can attenuate chronic ventricular remodeling after myocardial ischemia, we performed oral administration of CGA into murine myocardial ischemia models. The MI model was produced by permanent ligation of the left anterior descending coronary artery using an 8-0 suture passed under the arteries. Some MI mice were supplemented orally with CGA (30mg/kg/day, MI + CGA group) as a CGA-treated MI group, and other MI mice received vehicle (MI + vehicle group) as a vehicle-treated MI group. Sham-operated mice without MI also received vehicle (Sham + vehicle group) as a sham group, and sham-operated mice without MI received CGA (30mg/kg/day, Sham + CGA group, n = 8) as a Sham + CGA group. Just before sacrifice on day 14, we measured blood pressure, heart rate, and echocardiogram. We revealed the vehicle-treated MI group showed significantly impaired left ventricular contraction compared to the sham-operated group. However, the CGA-treated MI group showed significantly

improved ventricular contraction compared to the vehicle-treated MI group. Severe myocardial fibrosis with enhanced macrophage infiltration was observed in the vehicle-treated ischemia group. CGA attenuated these fibrotic changes with suppressed macrophage infiltration without systemic adverse effects. We concluded that CGA might effectively suppress chronic ventricular remodeling after myocardial ischemia because it is critically involved in the suppression of macrophage infiltration [20].

5. CGA ON MYOCARDITIS

Myocarditis is another serious myocardial disease. Patients with myocarditis in its severest form may suffer from rapidly progressive heart failure, shock, or arrhythmia [21-26]. In patients with myocarditis, autoimmune disease is considered to be responsible for the pathogenesis [27-29]. Autoimmune myocarditis can be induced in mice or rats by immunization with cardiac myosin [27, 28, 30]. This model revealed that reactive oxygen species (ROS) and inflammation are key modulators of myocarditis [28, 31, 32].

A cell adhesion glycoprotein molecule, intercellular adhesion molecule (ICAM)-1 revealed upregulation of ligand expression by inflammatory cytokines as an important switch to initiate adhesion [33]. It has shown that ICAM-1 associates pathological process of myocarditis [34, 35]. It was reported that ICAM-1 in myocardial cells plays a critical role in the acute viral myocarditis that investigated using ICAM-1 neutralizing antibody [34]. Resent data also revealed that ICAM-1 is expressed by inflammation and ROS [36, 37]. Therefore, it suggests that ICAM-1 is required for the onset of myocarditis. To investigate the effect of CGA on myocarditis, we used a murine model of experimental autoimmune myocarditis (EAM). Balb/c mice were immunized with cardiac myosin peptides and complete Freund's adjuvant. CGA or vehicle (Cont) was administered orally from day 0 to day 21 ($n = 6$ and 7, respectively) and the animals were sacrificed on day 21. CGA significantly suppressed ICAM-1 expression in the EAM hearts. The suppressed ICAM-1 tended to reduce myocardial fibrosis compared to control EAM hearts. The findings suggest CGA influences inhibition of cell adhesion in myocarditis, and may have beneficial effects on the progression of myocarditis [38].

CONCLUSION

Our findings and previous data suggest that the CGA treatment may have beneficial effects on the progression of myocardial diseases.

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REFERENCES

[1] Bouayed, J.; Rammal, H.; Dicko, A.; Younos, C.; Soulimani, R. Chlorogenic acid, a polyphenol from Prunus domestica (Mirabelle), with coupled anxiolytic and antioxidant effects. *J. Neurol. Sci.* 2007, 262, 77-84.

[2] Granado-Serrano, A.B.; Martin, M.A.; Izquierdo-Pulido, M.; Goya, L.; Bravo, L.; Ramos, S. Molecular mechanisms of (-)-epicatechin and chlorogenic acid on the regulation of the apoptotic and survival/ proliferation pathways in a human hepatoma cell line. *J. Agric. Food. Chem*. 2007, 55, 2020-7.

[3] Feng, R.; Lu, Y.; Bowman, L.L.; Qian, Y.; Castranova, V.; Ding, M. Inhibition of activator protein-1, NF-kappaB, and MAPKs and induction of phase 2 detoxifying enzyme activity by chlorogenic acid. *J. Biol. Chem*. 2005, 280**,** 27888-95.

[4] Xu, Y.; Chen, J.; Yu, X.; Tao, W.; Jiang, F.; Yin, Z.; Liu, C. Protective effects of chlorogenic acid on acute hepatotoxicity induced by lipopolysaccharide in mice. *Inflamm. Res*. 2010, 59**,** 871-7.

[5] Chauhan, P.S.; Satti, N.K.; Sharma, P.; Sharma, V.K.; Suri, K.A.; Bani, S. Differential effects of chlorogenic acid on various immunological parameters relevant to rheumatoid arthritis. *Phytother. Res.* 2012, 26**,** 1156-65.

[6] Panagiotakos, D.B.; Pitsavos, C.; Chrysohoou, C.; Kokkinos, P.; Toutouzas, P.; Stefanadis, C. The J-shaped effect of coffee consumption

on the risk of developing acute coronary syndromes: the CARDIO2000 case-control study. *J. Nutr.* 2003, 133, 3228-32.

[7] Happonen, P.; Voutilainen, S.; Salonen. J.T. Coffee drinking is dose-dependently related to the risk of acute coronary events in middle-aged men. *J. Nutr.* 2004, 134, 2381-6.

[8] Andersen, L.F.; Jacobs, Jr. D.R.; Carlsen, M.H.; Blomhoff, R. Consumption of coffee is associated with reduced risk of death attributed to inflammatory and cardiovascular diseases in the Iowa Women's Health Study. *Am. J. Clin. Nutr.* 2006, 83, 1039-46.

[9] Watanabe, T.; Arai, Y.; Mitsui, Y.; Kusaura, T.; Okawa, W.; Kajihara, Y.; et al. The blood pressure-lowering effect and safety of chlorogenic acid from green coffee bean extract in essential hypertension. *Clin. Exp. Hypertens.* 2006, 28, 439-49.

[10] Ochiai, R.; Jokura, H.; Suzuki, A.; Tokimutsu, I.; Ohishi, M; Komai, N., et al. Green coffee bean extract improves human vasoreactivity. *Hypertens. Res.* 2004, 27, 731-7.

[11] Suzuki, A.; Yamamoto, N.; Jokura, H.; Yamamoto, M.; Fujii, A.; Tokimitsu, I.; et al. Chlorogenic acid attenuates hypertension and improves endothelial function in spontaneously hypertensive rats. *J. Hypertens.* 2006, 24, 1065-73.

[12] Wu, H.Z.; Luo, J.; Yin, Y.X.; Wei, Q. Effects of chlorogenic acid, an active compound activating calcineurin, purified from Flos Lonicerae on macrophage. *Acta. Pharmacol. Sin.* 2004, 25, 1685-9.

[13] Velagaleti, R.S.; Pencina, M.J.; Murabito, J.M.; Wang, T.J.; Parikh, N.I.; D'Agostino, R.B.; Levy, D.; Kannel, W.B.; Vasan, R.S. Long-term trends in the incidence of heart failure after myocardial infarction. *Circulation*. 2008, 118, 2057-62.

[14] Onai, Y.; Suzuki, J.; Maejima, Y.; Haraguchi, G.; Muto, S.; Itai, A.; Isobe, M. Inhibition of NF-kappaB improves left ventricular remodeling and cardiac dysfunction after myocardial infarction. *Am. J. Physiol. Heart. Circ. Physiol.* 2007, 292, H530-8.

[15] Brasier, A.R. The nuclear factor-kappab-interleukin-6 signalling pathway mediating vascular inflammation. *Cardiovasc. Res.* 2010, 86, 211-8.

[16] Frangogiannis, N.G.; Smith, C.W.; Entman, M.L. The inflammatory response in myocardial infarction. *Cardiovasc. Res.* 2002, 53, 31-47.

[17] Kempf, T.; Zarbock, A.; Vestweber, D.; Wollert, K.C. Anti-inflammatory mechanisms and therapeutic opportunities in myocardial infarct healing. *J. Mol. Med. (Berl).* 2012, 90, 361-369.

[18] Vilahur, G.; Juan-Babot, O.; Pena, E.; Onate, B.; Casani, L.; Badimon, L. Molecular and cellular mechanisms involved in cardiac remodeling after acute myocardial infarction. *J. Mol. Cell. Cardiol.* 2011, 50, 522-533.

[19] Vanhoutte, D.; Schellings, M.; Pinto, Y.; Heymans, S. Relevance of matrix metalloproteinases and their inhibitors after myocardial infarction: A temporal and spatial window. *Cardiovasc. Res.* 2006, 69, 604-613.

[20] Kanno, Y.; Watanabe, R.; Zempo, H.; Ogawa, M.; Suzuki, J.; Isobe, M. Chlorogenic acid attenuates ventricular remodeling after myocardial infarction in mice. *Int. Heart. J.* 2013, 54**,** 176-80.

[21] Zee-Cheng, C.S.; Tsai, C.C.; Palmer, D.C.; Codd, J.E.; Pennington, D.G.; Williams, G.A. High incidence of myocarditis by endomyocardial biopsy in patients with idiopathic congestive cardiomyopathy. *J. Am. Coll. Cardiol*. 1984, 3**,** 63-70.

[22] Oakley, C.M. Myocarditis, pericarditis and other pericardial diseases. *Heart*. 2000, 84**,** 449-54.

[23] Batra, A.S.; Lewis, A.B. Acute myocarditis. *Curr. Opin. Pediatr*. 2001, 13**,** 234-9.

[24] Haas, G.J. Etiology, evaluation, and management of acute myocarditis. *Cardiol. Rev*. 2001, 9**,** 88-95.

[25] Liu, P.P.; Mason, J.W. Advances in the understanding of myocarditis. *Circulation*. 2001, 104**,** 1076-82.

[26] Frustaci, A.; Chimenti, C.; Calabrese, F.; Pieroni, M.; Thiene, G.; Maseri, A. Immunosuppressive therapy for active lymphocytic myocarditis: virological and immunologic profile of responders versus nonresponders. *Circulation*. 2003, 107**,** 857-63.

[27] Pummerer, C.L.; Luze, K.; Grassl, G.; Bachmaier, K.; Offner, F.; Burrell, S.K.; Lenz, D.M.; Zamborelli, T.J.; Penninger, J.M.; Neu, N. Identification of cardiac myosin peptides capable of inducing autoimmune myocarditis in BALB/c mice. *J. Clin. Invest*. 1996, 97**,** 2057-62.

[28] Suzuki, J.; Ogawa, M.; Futamatsu, H.; Kosuge, H.; Sagesaka, Y.M.; Isobe, M. Tea catechins improve left ventricular dysfunction, suppress myocardial inflammation and fibrosis, and alter cytokine expression in rat autoimmune myocarditis. *Eur. J. Heart. Fail*. 2007, 9**,** 152-9.

[29] Suzuki, J.; Ogawa, M.; Watanabe, R.; Morishita, R.; Hirata, Y.; Nagai, R.; Isobe, M. Autoimmune giant cell myocarditis: clinical

characteristics, experimental models and future treatments. *Expert. Opin. Ther. Targets*. 2011, 15**,** 1163-72.

[30] Ashigaki, N.; Suzuki, J.; Ogawa, M.; Watanabe, R.; Aoyama, N.; Kobayashi, N.; Hanatani, T.; Sekinishi, A.; Zempo, H.; Tada, Y.; Takamura, C.; Wakayama, K.; Hirata, Y.; Nagai, R.; Izumi, Y.; Isobe, M. Periodontal bacteria aggravate experimental autoimmune myocarditis in mice. *Am. J. Physiol. Heart. Circ. Physiol*. 2013, 304**,** H740-8.

[31] Ellis, C.R.; Di Salvo, T. Myocarditis: basic and clinical aspects. *Cardiol. Rev*. 2007, 15**,** 170-7.

[32] Kishimoto, C.; Nimata, M.; Okabe, T.A.; Shioji, K. Immunoglobulin treatment ameliorates myocardial injury in experimental autoimmune myocarditis associated with suppression of reactive oxygen species. *Int. J. Cardiol*. 2013, 167**,** 140-5.

[33] Long, E.O. ICAM-1: getting a grip on leukocyte adhesion. *J. Immunol*. 2011, 186**,** 5021-3.

[34] Seko, Y.; Matsuda, H.; Kato, K.; Hashimoto, Y.; Yagita, H.; Okumura, K.; Yazaki, Y. Expression of intercellular adhesion molecule-1 in murine hearts with acute myocarditis caused by coxsackievirus B3. *J. Clin. Invest*. 1993, 91**,** 1327-36.

[35] Nakagawa, P.; Liu, Y.; Liao, T.D.; Chen, X.; Gonzalez, G.E.; Bobbitt, K.R.; Smolarek, D.; Peterson, E.L.; Kedl, R.; Yang, X.P.; Rhaleb, N.E.; Carretero, O.A. Treatment with N-acetyl-seryl-aspartyl-lysyl-proline prevents experimental autoimmune myocarditis in rats. *Am. J. Physiol. Heart. Circ. Physiol*. 2012, 303**,** H1114-27.

[36] Kim, Y.S.; Ahn, Y.; Hong, M.H.; Joo, S.Y.; Kim, K.H.; Sohn, I.S.; Park, H.W.; Hong, Y.J.; Kim, J.H.; Kim, W.; Jeong, M.H.; Cho, J.G.; Park, J.C.; Kang, J.C. Curcumin attenuates inflammatory responses of TNF-alpha-stimulated human endothelial cells. *J. Cardiovasc. Pharmacol*. 2007, 50**,** 41-9.

[37] Phalitakul, S.; Okada, M.; Hara, Y.; Yamawaki, H. Vaspin prevents TNF-alpha-induced intracellular adhesion molecule-1 via inhibiting reactive oxygen species-dependent NF-kappaB and PKCtheta activation in cultured rat vascular smooth muscle cells. *Pharmacol. Res*. 2011, 64**,** 493-500.

[38] Zempo, H.; Suzuki, J.; Ogawa, M.; Watanabe, R.; Tada, Y.; Takamura, C.; Isobe, M. Chlorogenic acid suppresses a cell adhesion molecule in experimental autoimmune myocarditis in mice. *Immun. Endoc. Metab. Agents in Med. Chem.* 2013, 13, 232-236.

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Chapter 4

THE ROLE OF DIET IN THE COMPOSITION AND ACTIVITY OF GUT MICROBIOTA

Lucia Birošová [1] and Petra Olejníková [2]
[1]Department of Nutrition and Food Quality Assessment,
Faculty of Chemical and Food Technology,
Slovak University of Technology, Bratislava, Slovakia
[2]Institute of Biochemistry and Microbiology,
Faculty of Chemical and Food Technology,
Slovak University of Technology, Bratislava, Slovakia

ABSTRACT

Gut microbiota represents an important part in food digestion, immunity, metabolism and gene expression as well. It consists of about 10^{14} bacterial cells represented by more than 400 species that can affect its host in many ways. Qualitative and quantitative composition of microbiota can be influenced by many factors such stress, age, antibiotic therapy or diet. The last mentioned factor is a study subject of many researchers nowadays. Many studies have pointed out that diet based on vegetables such vegan diet shift human gut microbiota to higher prevalence of beneficial bacteria and consequently to healthier lifestyle. In our research we have studied qualitative and quantitative composition of cultivable bacterial genera in stool of 240 healthy volunteers aged between 20 and 60 differ in diet. More than half (136) belongs to vegetarians divided to semi vegetarians (pullo and pesco), lacto

vegetarians, lacto-ovo vegetarians and vegans. No significant differences were found between meat-eaters and vegetarians. On the other hand, lacto-, vegans had lower number of almost all studied bacterial genera. We have also focused on presence of potential mutagenic compounds in stool. Percentage of stools positive on potential mutagenic compounds was almost same in both groups. However, obesity is also linked to shifts in microbial composition, obese and lean subjects were also studied. Obese subjects have only slightly increased staphylococci. Furthermore, we have observed more differences between underweight and lean subjects.

Keywords: microbiota, vegetarians, obesity, diet

INTRODUCTION

Human intestinal microbiota is crucial for its host to perform many functions for proper physiology and metabolism. Many mutual beneficial and cooperative interactions between human gut microbes and its host have been observed. As microbial activity affects human functions especially digestion in many ways, gut microbiota is sometimes considered as a virtual organ (O´Hara and Shanahan, 2006). A Large spectrum of bacteriostatically and microbicidally acting substances are produced by intestinal microorganisms whereby forms microbial barrier against implantation and augmentation of pathogenic and potential pathogenic organisms (Zimmer et al., 2012). Beside this so called "colonisation resistance," microbiota also affects homeostasis, function of, immune system or digestion (Browne et al., 2016). On the other hand, many factors such age (Saraswati and Sitaraman, 2015), antibiotic therapy (Panda et al., 2014), stress (Bailey et al., 2011) or diet (Zimmer et al., 2012) might influence composition of intestinal microbiota and thereby the whole physiology or development of pathological processes in individuals. Among these factors, diet is a major factor influencing the composition and metabolism of the colonic microbiota. Diet can highly impact the gut environment, including gut transit time and pH; and changing the intake of the three main macronutrients (carbohydrates, proteins and fats) can significantly affect the composition of the microbiota (Scott et al., 2013). Intestinal bacteria also produce enzymes ubiquitous for degradation of the structural polysaccharides found in dietary plant material and relies on the colonic microbiota for efficient degradation of recalcitrant plant cell walls. The main forms of carbohydrates available to bacteria in the colon include resistant

starch, non-starch polysaccharides and oligosaccharides. These compounds are fermented by specialized bacteria such *Ruminococcus* sp. resulted in production of short chain fatty acids which affect host metabolism and immunity (Scott et al., 2013). During past decades, vegetarian diet has increased its popularity as a healthy and potentially therapeutic dietary choice (Craig, 2009). Generally vegetarian diet is linked with many health benefits, such improving insulin resistance, lowering diabetes or cardiometabolic disease risk, hypertension or some cancers (Glick-Bauer and Ming-Chin, 2014). Some studies pointed out linkage between dietary patterns and gut microbiota profiles. Wu et al. (2011) proposed 2 enterotypes associated with diet; the enterotype with dominancy of *Bacteroides* associated with high fat and animal protein diet and *Prevotella* enterotype linked with carbohydrate and vegetarian diet. Western diet is also characterized by predominance of *Bacteroides* enterotype and greater abundance of gram-positive bacteria, mainly Firmicutes. On the other hand, both *Bacteroides* and *Prevotella* are characterized as polysacharide-degrading bacteria (Kim et al., 2013). Thus the associations between diet and microbiota composition may vary depending on whether researchers are conducting global assessments of gut microbiota or seeking greater taxonomic detail through DNA sequencing (Glick-Bauer and Ming-Chin, 2014). Some other beneficial bacterium *Faecalibacterium prausnitzii* was detected in higher ratio in vegans. This bacterium belongs to butyrate producer in the class *Clostridia* (Firmicutes) and display anti-inflammatory effect and protective role for colonocytes (Kim et al., 2013). Intestinal microbial composition of meat-eaters used to be enriched in *Bifidobacterium*, *Peptococcus* and *Lactobacillus* compared to vegans (Reddy et al., 1975; van Fassen et al., 1987). These bacteria are able to *de novo* synthesize and supply some B-group vitamins. From this point of view, some studies pointed out deficiency of B12 in vegans, leading to anaemia (Refsum et al., 2001; Allen and Stabler, 2008).

It is suggested that the gut microbiota play an important role in the regulation of energy balance and weight in animals and humans and may influence the development and progression of obesity (DiBaise et al., 2012). The role of the Western diet in promoting an obesogenic gut microbiota is being confirmed in subjects (Musso et al., 2010). Obesity has been linked to decreased prevalence of Bacteroidetes, and increased in Firmicutes and Actinobacteria. Study performed on mouse model with a humanized gut microbiome indicated rapid shift (only one day) in microbial composition after switching from a low-fat, plant polysaccharide-rich diet to a high-fat, high-sugar Western diet (Turnbaugh et al., 2009). These rapid changes in gut

microbiota induced by short-term alterations in nutrient load were observed only in lean, but not in obese humans (DiBaise et al., 2012).

In this study, we have compared qualitative and quantitative composition of gut microbiota in healthy individuals divided according to diet. At first we were interested in differences between vegetarians and meat-eaters, but we have also focused on the effect of obesity on the composition and shifts in intestinal microbiota. The fact is that altered diet can affect metabolic activity of gut microbiota, but we have also examined the prevalence of cases with potential mutagenic activity.

MATERIAL AND METHODS

Study Design and Subjects

Healthy human subjects were recruited in Bratislava (Slovakia) and its surrounding area. All 136 volunteers were born and raised in Slovakia. 104 volunteers with common diet (meat-eaters). Group of vegetarians consisted of 40 vegans, 60 lacto-ovo-vegetarians and 36 semi-vegetarians with the distribution of BMI >16. (Table 3) According to the dietary questionnaires the daily dietary fibre intake of vegetarians was higher than that in group of meat eaters. Admission of vitamins, minerals and trace elements by subjects was only in natural form (no supplements). Age of all subjects ranged from 20 to 60 years.

Lacto-ovo-vegetarians did not consume meat in any form, but ate animal products such as milk, cheese and eggs. A vegan diet was considered for those who did not consume animal products. Semi-vegetarians have stated that they consume animal products and also poultry and fish meat. The omnivorous consumed all type of meat (pork, red meat, poultry and fish) minimal 5 times a week. All volunteers were healthy, and had not undergone antibiotic or other medical therapy in last 6 months. Faecal samples were collected from April to May.

Sample Collection and Microbiological Analysis

Fresh faecal samples were collected and transferred to the laboratory in sterile collection tubes. Colon microbiota was assayed by culture method on selective diagnostic media according to Mitsuoka and Hayakawa (1972). Fresh

faecal samples were weight (1g), suspended in 10 ml BHI (Brain Heart Infusion) medium and series of 10-fold dilutions (10^{-1} to 10^{-8}) was prepared. For total anaerobes and aerobes determination, 2 nonselective growth media were used: modified medium 10 (M10) for anaerobes, Trypticase soy (TS) agar (Biolife Italiana srl., Milan, Italy) for aerobes. Moreover 16 selective growth media such as yeast extract glucose chloramphenicol (YGC) agar (OXOID Deutschland GmBH., Wesel, Germany) for yeasts and fungi, neomycin-brilliant green-taurocholate-blood (NBGT) growth media for Bacteroidetes, neomycin-Nagler (NN) growth media for lecithinase-positive clostridia, modified veilonella selective (VS) growth media for *Veilonellae* spp., eosin methylene blue (EMB) agar (Biolife Italiana srl.), laurylsuphate with MUG (LS MUG) agar (Biolife Italiana srl.) and violet red bile lactose (VRBL) growth media (Biolife Italiana srl.) for *Enterobacteriaceae*, xylose lysine deoxycholate (XLD) growth media (Biolife Italiana srl.) for *Salmonella* spp detection., Baird-Parker (BP) growth media (Biolife Italiana srl.) for *Staphylococcus aureus*, 110 medium (110) growth media (Biolife Italiana srl.) for total staphylococci, Slanetz-Bratley (SB) growth media (Biolife Italiana srl.) for enterococci, PALCAM (PC) agar (Biolife Italiana srl.) for *Listeria* spp., thioglycollate (TG) agar (Biolife Italiana srl.) for clostridia Rogosa (RG), microinoculum (MI) MRS growth media (Biolife Italiana srl.) for lactobacilli and bifidobacteria). Growth media for anaerobic cultivation M10, NN, VS, NBGT were prepared in laboratory according Mitsuoka and Hayakawa (1972). For determination of anaerobes NN, RG, NBGT, TG for 2 days and M10, VS growth media were cultivated in anaerobic chamber (Bactron I, Sheldon Manufacturing, Inc., Cornelius, Oregon, USA) for 4 days at 37°C. Agar plates containing growth media TS, BP, EMB, LS, VRBL, XLD were cultivated aerobically at 37°C for 24h and PC, 110 for 48h. SB plates were cultivated aerobically at 45°C for 48h and YGC at 25°C for 5 days.

Assessment of Potential Mutagenicity

Ames incorporation method was used for detection of potential mutagens in faecal samples. Potential mutagenic activity was performed using Ames test without metabolic activation using *Salmonella* Typhimurium TA 98 (CCM 3811) and TA 100 (CCM 3812) (obtained from Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic). Briefly, 1g of fresh faecal samples was suspended in 10 ml BHI medium and sterilized through filtration (0.22 μm membranes, GH Polypro, VWR, Wien, Austria).

Filtrated sample was applied in Ames test. Positive response was defined as a reproducible, two-fold increase of *Salmonella* revertants compared to spontaneous revertants. As a positive mutagen 3-(5-nitro-2furyl) acrylic acid (NFAA, Slovakofarma, Hlohovec, Slovakia) was used. The results from Ames test represent the mean of three separate experiments, which were statistically evaluated using Student´s t-test.

RESULTS AND DISCUSSION

Differences in Gut Microbiota Composition According to Vegetable Diet

Connection between plant diet and gut microbiota composition have been shown by many studies (vanFassen et al., 1987; Zimmer et al., 2012; DiBaise et al., 2012), but this phenomena is still not fully understood. In our study we have compared faecal microbiota of randomly chosen 240 healthy subjects. According to the diet habits, 104 volunteers belonged to meat-eaters and 136 were vegetarians (vegans, lacto and lacto-ovo-vegetarians, semi-vegetarians). Table 1 shows differences in quantitative composition of faecal microbiota between different groups of vegetarians and people with conventional middle - east European diet (all types of meat more than 4 times a week, balanced diet). It is evident that the count of total aerobes and anaerobes was decreased in vegan samples though standard error was too high because of high discrepancies in this group. Lower bacterial rate could also be caused by high admission of indigestible dietary fibre by vegans and it is possible that it captures and takeaway the colon microbiota bacteria. In samples of vegans we have observed lower number of total clostridia. This findings correlate with data found by Liszt et al., (2009). This study found out that vegetarian diet affects intestinal microbiota predominantly by decreasing of clostridia. Beside clostridia in this group we have detected lower number of *Veilonella* spp., and no staphylococci compared to other studied groups (Table 1). On the other hand, vegans and semi-vegetarians obtained higher number of *Listeria* spp. and Bacteroidetes. Although we have identified only class and not genus, our results are not in accordance with conclusions of some other studies (Wu et al., 2011; Ruengsomwong et al., 2014) wherein *Prevotella* enterotype has dominated in vegan group. From Table 1 is evident that no significant differences in count of studied bacteria were observed between lacto-ovo-vegetarians, semi-vegetarians and meat-eaters. This could be also caused by

fact, that we have applied only conventional cultivation method for detection of bacterial classes and not genus nor strains. On the other hand, in our previous study (Šakova et al., 2015) we have shown that subjects with same qualitative and quantitative profile of cultivable bacteria differs in separation patterns for different microbial populations obtained after DGGE analysis. Even the culturing dependent technique for evaluation of gut microbiota was considered as inadequate, the most recent study with a complete novel workflow observed a strong correlation between uncultured and culture dependent approaches at the species level. The results of Browne et al., (2016) demonstrate that a considerable proportion of the bacteria within the faecal microbiota can be cultured even if using only one complex growth medium. Studies that evaluated the composition of gut microbiota using cultivation dependent and independent methods showed that the most predominant microorganisms are sporulating bacteria (*Clostridium* cluster XIVa and *Clostridium* cluster IV) (Browne et al., 2016).

Table 1. Quantitative analyses on faecal flora (log_{10} CFU/g stool) according diet after cultivation of faecal samples on selected diagnostic growth media

Bacteria	vegans	Lacto-ovo-vegetarians	Semi-vegetarians	Meat-eaters
Total aerobes	5.80 ± 3.07	8.24 ± 0.26	8.91 ± 0.25	8.36 ± 0.17
Total anaerobes	5.25 ± 2.78	8.21 ± 0.30	9.09 ± 0.22	8.41 ± 0.17
Bacteroidetes	5.51 ± 0.35	3.46 ± 0.26	3.46 ± 0.46	3.20 ± 0.22
Lactic acid bacteria	5.83 ± 1.42	5.61 ± 0.31	6.00 ± 0.24	6.17 ± 0.21
Total clostridia.	5.86 ± 3.06	8.55 ± 0.26	8.35 ± 0.46	8.63 ± 0.14
Lecithinase positive *Clostridium* spp.	1.80 ± 0.8	0.80 ± 0.24	1.19 ± 0.38	1.32 ± 0.21
Enterobacteriaceae	6.78 ± 0.97	7.32 ± 0.24	7. 58 ± 0.30	7.48 ± 0.16
Enterococcus spp.	7.26 ± 1.23	6.69 ± 0.35	7.40 ± 0.28	6.63 ± 0.18
Staphylococcus spp.	4.70 ± 0.12	4.19 ± 0.39	5.44 ± 0.34	5.88 ± 0.29
Staphylococcus aureus	ND	0.97 ± 0.24	0.97 ± 0.32	1.06 ± 0.19
Listeria spp.	4.78 ± 0.31	2.90 ± 0.33	4.24 ± 0.43	2.64 ± 0.24
Veilonella spp.	5.88 ± 0.11	7.16 ± 0.31	6.91 ± 0.27	7.15 ± 0. 23
Yeasts and Fungi	3.65 ± 1.95	4.15 ± 0.21	3.75 ± 0.38	3.89 ± 0.18

ND – not detected.

Potential Mutagenic Activity of Vegetarians and Meat-Eaters Stool

Metabolic activity of gut bacteria leads to production of different substances such as short chain fatty acids or bacteriocins but it can also contribute to generation of carcinogenic metabolites and inflammation. For example, probiotic lactobacilli protect the gut environment against genotoxic and carcinogenic effects of heterocyclic aromatic amines (HA) originating from fried food, whereas some species of Bacteroidetes appear to contribute to the conversion of HA to DNA-reactive carcinogens (Kassie et al., 2004). For determination of potential mutagenic activity of stool samples, we have applied incorporation Ames test with two bacterial strains of *Salmonella* Typhimurium TA98 (for detection of point mutations) and TA100 (for detection of frameshift mutations). Table 2 shows the highest percentage of stools with potential mutagenic activity was observed in group of vegans. Mutagenic activity has been detected only in case of *S.* Typhimurium TA98. The ratio of potential mutagenic samples detected by both applied strains was equal in lacto-ovo-vegetarian samples. Almost 4% samples account positivity in Ames test with TA98 as well as with TA100. With the introduction of meat to diet, percentage of samples with positivity using TA100 has increased and with TA98 decreased. Also number of combined positivity has risen. As it has been correlated in our previous study (Šaková et al., 2015) presence of potentially mutagenic substances in the colon is related to the presence of particular bacteria of the genus Bacteroidetes and in minor range also lecithinase-positive clostridia.

Table 2. Percentage of samples positive on potential mutagenic activity in Ames test

Ames strain	Vegans	Lacto-ovo-vegetarians	Semi-vegetarians	Meat-eaters
TA98 (point mutation)	33.3%	21.15%	8.6%	14.4%
TA100 (frameshift mutation)	0%	21.15%	29.6%	26.9%
TA98 + TA100 (both mutation detected)	0%	3.8%	6.1%	5.8%

Differences in Gut Microbiota Composition According to Obesity

Although some studies show linkage between obesity and composition of intestinal microbiota we have also studied bacterial differences between groups divided according to subject's body mass index (BMI). Table 3 shows number of studied bacterial classes in underweight (8 volunteers), lean (141 volunteers), overweight (61 volunteers) and obese (30 volunteers) subjects. It is evident, that differences in counts were only moderate. Generally, the highest number of majority bacterial classes was observed in underweight group. Beside total aerobes, we have detected also the highest number of total clostridia, *Enterobacteriaceae* and staphylococci. Even though some studies imply (Musso et al., 2010) that reduction in Bacteroidetes for obese microbiome is typical, our data have shown no difference in number of these bacteria. Researchers also asign, that obese humans have higher abundance of Firmicutes such lactobacilli, clostridia or staphylococci (DiBaise et al., 2012).

Table 3. Quantitative analyses on fecal flora (log_{10} CFU/g stool) according BMI after cultivation of fecal samples on selected diagnostic growth media

	Underweight BMI < 18.5	Normal BMI 18.5-25	Overweight BMI 25 - 30	Moderately obese BMI 30 - 35
Average BMI	17.20 ± 0.15	21.80 ± 0.15	27.30 ± 0.18	32.80 ± 0.65
Total aerobes	7.08 ± 0.93	6.37 ± 0.14	6.01 ± 0.21	6.05 ± 0.29
Total anaerobes	6.96 ± 0.85	6.43 ± 0.13	6.24 ± 0.22	6.09 ± 0.32
Bacteroidetes	2.79 ± 0.63	2.16 ± 0.10	2.27 ± 0.17	2.60 ± 0.29
Lactic acid bacteria	5.42 ± 0.25	5.17 ± 0.14	4.67 ± 0.23	5.01 ± 0.37
Total clostridia	7.23 ± 0.94	6.65 ± 0.13	6.07 ± 0.21	5.82 ± 0.23
Enterobacteriaceae	6.06 ± 0.54	5.74 ± 0.14	5.61 ± 0.22	5.26 ± 0.30
Enterococcus spp.	4.70 ± 0.62	4.90 ± 0.14	4.45 ± 0.25	5.16 ± 0.44
LP clostridia	0.32 ± 0.22	0.64 ± 0.09	0.47 ± 0.15	0.63 ± 0.19
Listeria spp.	2.00 ± 0.66	1.47 ± 0.12	1.33 ± 0.18	1.51 ± 0.29
Staphylococcus spp.	4.96 ± 0.86	3.35 ± 0.17	3.85 ± 0.38	4.22 ± 0.52
Staphylococcus aureus	0.50 ± 0.36	0.54 ± 0.09	0.50 ± 0.12	0.39 ± 0.16
Veillonella spp.	5.43 ± 0.49	5.31 ± 0.16	5.17 ± 0.23	5.52 ± 0.44
Yeasts and Fungi	2.13 ± 0.56	2.35 ± 0.11	2.29 ± 0.13	2.25 ± 0.30

Our results affirm only higher number of staphylococci, compared to lean subjects. On the other hand, we have observed higher number of staphylococci also in group of underweight volunteers.

CONCLUSION

Emerging evidence shows that diet plays an important role in abundance and composition of intestinal microbiota. This work represents comparison of faecal microbiota of people on predominantly plant diet and meat-eaters. Our results show lower counts of total cultivable aerobes and anaerobes as well as clostridia in vegans. Other differences were not significant. Majority of vegan stool samples were positive in Ames test using *S. Typhimurium* TA98 strain. With the introduction of meat in diet, percentage of these samples has decreased, but positivity was detected also with strain TA100. Obesity as consequence of high fat diet is also linked with the shifts in microbiota composition. Comparison of subjects according to BMI showed more differences between underweight and lean subjects than between obese and lean ones. On the base of these data it is evident that mile-east European diet have well balanced intake of meat and plant cell food matrices.

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REFERENCES

Allen, R. H., Stabler, S. P., (2008) Identification and quantification of cobalamin analogues in human feces. *Am. J Clin. Nutr.* 87(5), 1324-35.

Maron, D., Ames, B., (1983) Revised methods for the *Salmonella* mutagenicity test. *Mut. Res.* 113, 173 – 215.

Bailey, T. M., Dowd, S. E., Galley, J. D., Hufnagle, A.R., Allen, R.G., Lyte, M., (2011) Exposure to a social stressor alters the structure

of the intestinal microbiota: Implications for stressor-induced immunomodulation. *Brain Behav. Immun*. 25(3), 397-407.

Browne, H. P., Forster, S. C., Anonye, B. O., Kumar, N., Neville, B. A., Stares, M. D., Goulding, D., Lawley, T.D., (2016) Culturing of ´unculturable´ human microbiota reveals novel taxa and extensive sporulation. *Nature* 533, 543-546.

Craig, W.J., (2009) Health effects of vegan diets, *Am. J. Clin. Nutr*. 89, 1627S-1633S.

DiBaise, J. K., Frank, D. N., Mathur, R., (2012) Impact of the gut microbiota on the development of obesity: Current concepts. *Amm. J. Gastroenterol.* Suppl. 1(1), 22-27.

Glick-Bauer, M., Ming-Chin, Y., (2014) The health advantage of a vegan diet: Exploring the gut microbiota connection. *Nutrients* 6, 4822-4838.

Kassie, F., Lhoste, E.F., Bruneau, A., Zsivkovits, M., Ferk, F., Uhl, M., Zidek, T., Knasmuller, S., (2004) Effect of intestinal microfloras from vegetarians and meaeaters on the genotoxicity of 2-amino-3methylimidazo (4,5f)quinoline, a carcinogenic heterocyclic amine. *J. Chromatogr. B* 302, 211-215.

Liszt, K., Zwielehner, J., Handschur, M., Hippe, B., Thaler, R., Haslberger, A.G., (2009) Characterization of bacteria, clostridia and Bacteroides in faeces of vegetarians using qPCR and PCR-DGGE fingerprinting. *Ann. Nutr. Metab*. 54, 253–257.

Mitsuoka, T., Hayakawa, K., (1972) Die Zusammensetzung der Faecalflora der verschiedenen Altersgruppen. *Die Faecalflora bei Menschen 223*, 333-342.

Musso, G., Gambino, R., Cassader, M., (2010) Obesity, diabetes and gut microbiota. *Diabetes care* 33(10), 2277-2284.

O´Hara, A.M., Shanahan, F., (2006) The gut flora as a forgotten organ. *EMBO Rep* 7, 688-93.

Panda, S., El khader, I., Casellas, F., Vivancos, J. L., Cors, M.G., Santiago, A., Cuenca, S., Guarner, F., Manichanh, Ch., (2014) Short-Term Effect of Antibiotics on Human Gut Microbiota. *PloS ONE* 9(4), e95476.

Reddy, B. S., Weisburgger, J. H., Wynder, E. L., (1975) Effects of high risk and low risk diets for colon carcinogenesis on fecal microflora and steroids in man. *J. Nutr.* 105, 878-884.

Refsum, H., Yanik, C. S., Gadkari, M., Schneede, J., Vollset, S. E., Orning, L., Guttormsen, A. B., Joglekar, A., Sayyad, M.G., Ulvik, A., Ueland, P. M., (2001) Hyperhomocysteinemia and elevated methylmalonic acid indicate

a high prevalence of cobalamin deficiency in Asian Indians. *Am. J. Clin. Nutr*. 74(2), 233-41.

Ruengsomwong, S., Korenori, Y., Sakamoto, N., Wannissorn, B., Nakayama, J., Nitisinprasert, S., (2014) Senior thai fecal microbiota comparison between vegetarians and non-vegetarians using pcr-dgge and real-time PCR. *J. Microbiol. Biotechnol*. 24, 1026-1033.

Scott, K. P., Gratz, S.W., Sheridan, P. O., Flint, H.J., Duncan, S. H., (2013) The influence of diet on the gut microbiota. *Pharm. Res*. 69(1), 52-60.

Saraswati, S., Sitaraman, R., (2015) Aging and the human gut microbiota—from correlation to causality. *Front. Microbiol*. 5: 764.

Turnbaugh, P. J., Ridaura, V. K., Faith, J. J., Rey, F. E., Knight, R., Gordon, J. I, (2009) The effect of diet on the human gut microbiome: a metageomic analysis in humanized gnotobiotic mice. *Sci. Transl. Med.* 1(6), 6ara14.

van Faasen, A., Bol, J., van Dokkum, W., Pikaar, N. A., Ockhuizen, T., Hermus, R. J. J., (1987) Bile acids, neutral steroids and bacteria in feces as affected by a mixed, a lacto-ovovegetarian, and a vegan diet. *Am. J. Clin. Nutr*. 46, 962-967.

Wu, G. D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y. Y., Keilbaugh, S. A., Bewtra, M., Knights, D., Walters, W.A., Knight, R., et al., (2011) Linking long-term dietary patterns with gut microbial enterotypes. *Science* 334, 105-108.

Zimmer, J., Lange, B., Frick, J-S., Sauer, H., Zimmermann, K., Schwiertz, A., Rusch, K., Klosterhalfen, S., Enck, P., (2012) A vegan or vegetarian diet substatially alters the human colonic faecal microbiota. *Eur. J. Clin. Nutr*. 66, 53-60.

BIOGRAPHICAL SKETCH

Lucia Birošová

Affiliation: Department of Nutrition and Food Quality Assessment, Faculty of Chemical and Food Technology, Slovak University of Technology

Education: In 2004 she graduated in Biochemistry and Biotechnology at the Faculty of Chemical and Food Technology STU in Bratislava, Slovakia and gained Ing. (MSc.). In 2008 she finished my Ph.D. in Microbiology at the Faculty of Chemical and Food Technology STU in Bratislava and gained scientific - academic degree PhD.

Business Address: Radlinského 9, 812 37 Bratislava, Slovakia

Research and Professional Experience:

Professional Appointments: She works in the area of microbiology for more than 10 years. After Ph.D. studies she has worked as a researcher at the Department of Biochemistry and Microbiology STU. For short time she has also worked at the Department of Experimental and Applied Genetics SMU. Currently, she is an associate professor and deputy head of the Department of Nutrition and Food Quality Assessment. In 2015 she habilitated in Microbiology at the University of Veterinary Medicine and Pharmacy in Košice, Slovakia. Her professional membership is in National Scientific Panel for Biological Hazards, Emerging Risks Expert Network EFSA, Czechoslovak Society for Microbiology. She is a member of the examination board. In 2011 she has gained ČSSM Award "Prize for the best Czech and Slovak microbiology" for her scientific results. In 2013 she has acquired RAJO award for best lecture at FIEP congress in section "Nutrition, movement activities and healthy active life style". Several times, she has also received a FEMS grant (Young Scientist Meeting Grant). Her research is focused mainly on the issue of antibiotic resistance in food and the environment. It also addresses the monitoring of the natural and synthetic substances biological effects, or the study of intestinal microbiota in relation to nutrition. She is the author or co-author of several scientific articles in peer-reviewed scientific journals and chapters in foreign monographs, co-author of two domestic monographs, as well as university textbooks. She participates in various grant projects as a principal investigator or co-worker.

Honors: ČSSM Award "Prize for the best Czech and Slovak microbiology"

RAJO award for best lecture at FIEP congress in section "Nutrition, movement activities and healthy active life style"

5x FEMS grant (Young Scientist Meeting Grant)

Publications Last 3 Years:

1. Zelená, V., Birošová L., Olejníková, P., Polák, M., Lakatoš, B., Varečka, Ľ.: Properties of anaerobic fungi isolated from several habitats: complexity of phenotypes, *General Physiology and Biophysics* 35(1), pp. 95-107, 2016
2. Šaková, N., Olejníková, P., Kaliňáková, B., Pangallo, D., Lakatoš, B., Nagyová, K., Valachovičová, B., Birošová, L.: Comparative study of

adult Slovak vegetarians and meat-eaters gut microflora, *J Food Nutrition Res* 54(3),pp. 260-269, 2015

3. Olejníková, P., Birošová, L., Švorc, L., Vihonská, Z., Fiedlerová, M., Marchalín, Š., Šafář, P.: Newly synthesized indolizine derivatives - antimicrobial and antimutagenic properties, *Chem. Pap.* 69(7), 983-992, 2015
4. Mackuľak, T., Birošová, L., Gál, M., Bodík, I., Grabic, R., Ryba, J., Škubák, J.: Wastewater analysis: the mean of the monitoring of frequently prescribed pharmaceuticals in Slovakia, *Environmental Monitoring and Assessment* 188 (1), 1 -12, 2016
5. Mackuľak, T., Vois, M., Grabic, R., Golovko, O., Vojs Staňová, A., Birošová, L., Medveďová, B., Híveš, J., Gál, M., Kromka, A., Hanusová, A.: Occurrence of pharmaceuticals, illicit drugs, and resistant types of bacteria in hospital effluent and their effective degradation by boron-doped diamond electrodes, *Monatsh Chem*.147(1), pp. 97-103, 2016
6. Mackuľak, T., Birošová, L., Bodík, I., Grabic, R., Takáčová, A., Smolinská, M., Hanusová, A., Híveš, J., Gál, M.: Zerovalent iron and iron (VI): effective means for the removal of psychoactive pharmaceuticals and illicit drugs from wastewaters, *Sci. Tot. Env*. 539 art. No. 18328, pp. 420-426, 2016
7. Mackuľak, T., Grabic, R., Gál, M., Gál, M., Birošová, L., Bodík, I.: Evaluation of different smoking habits during music festivals through wastewater analysis, *Env. Tox. Pharm.*40 (3), pp. 1015-1020, 2015
8. Mackuľak, T., Nagyova K., Grabic, R., Koba, O., Gál, M., Birošová, L.: Utilization of Fenton-like reaction for antibiotics and resistant bacteria elimination in different parts of WWTP, *Env. Tox. Pharm.* 40(2), pp. 492-497, 2015
9. Mackuľak, T., Birošová, L., Grabic, R., Škubák, J., Bodík, I: National monitoring of nicotine use in Czech and Slovak Republic based on wastewater analysis, *Env. Sci. Pol. Res.* 22(18), pp.14000-14006, 2015
10. Mackuľak, T., Mosný, M., Škubák, J., Grabic, R., Birošová, L.: Fate of psychoactive compounds in wastewater treatment plant and the possibility of their degradation using aquatic plants, *Env. Tox. Pharm.* 39(2), 969-973, 2015

11. Mackuľak, T., Mosný, M., Grabic, R., Golovko, O., Koba, O., Birošová, L.: Fenton-like reaction: A possible way to efficiently remove illicit drugs and pharmaceuticals from wastewater, *Env. Tox. Pharm.* 39 (2), 483-488, 2015
12. Mackuľak, T., Škubák, J., Grabic, R., Ryba, J., Birošová, L., Fedorova, G., Špalková, V., Bodík, I.: National study of illicit drug use in Slovakia based on wastewater analysis, *Sci. Tot. Env*. 494-495, 158-165, 2014
13. Birošová L., Mackuľak, T., Bodík, I., Ryba, J., Škubák, J., Grabic, R.: Pilot study of seasonal occurrence and distribution of antibiotics and drug resistant bacteria in wastewater treatment plants in Slovakia, *Science of the Total Environment* 490, 440-444, 2014
14. Žemlička, L., Fodran, P., Lukeš, V., Vagánek, A., Slováková, M., Staško, A., Dubaj, T., Karabín, M., Birošová, L., Rapta, P.: Physicochemical and biological properties of luteolin-7-O-glucoside (cynaroside) isolated from Anthriscus sylvestris (L.) Hoffm, *Monatsh Chem* 145 (8), 1307-1318, 2014

BIOGRAPHICAL SKETCH

Petra Olejnikova

Affiliation: Department of Biochemistry and Microbiology, Faculty of Chemical and Food Technology, Slovak University of Technology

Education: In 2002 she graduated in Biochemistry and Biotechnology at the Faculty of Chemical and Food Technology STU in Bratislava, Slovakia and gained Ing. (MSc.). In 2009 she finished her Ph.D. in Microbiology at the Faculty of Chemical and Food Technology STU in Bratislava and gained scientific - academic degree PhD.

Business Address: Radlinského 9, 812 37 Bratislava, Slovakia

Research and Professional Experience:

Professional Appointments: She has been in the area of microbiology for more than 13 years. Since 2005 she has been working as a researcher at the Department of Biochemistry and Microbiology STU. She is a member of Czechoslovak Society for Microbiology and since 2010 she is part of executive board. She has received a FEMS grant (Young Scientist Meeting

Grant) for several times. Her research is mainly focused on the field of microbiology orienting her work toward bacterial and fungal cells. She has experiences in microbial diagnosis and in field of fungal resistance and development of newly synthetized compounds as antimicrobial agents. Her current research covers the study of intestinal microbiota. She is the author or co-author of several scientific publications in peer-reviewed scientific journals and university textbooks as well. She participates in various grant projects as co-worker.

Honors 5x FEMS grant (Young Scientist Meeting Grant)

Publications Last 3 Years:

1. Zelená, V., Birošová, L., Olejníková, P., Polák, M., Lakatoš, B. and Varečka, Ľ. (2015). Properties of anaerobic fungi isolated from several habitats: complexity of phenotypes. *General physiology and biophysics*. IF 1,173.
2. Olejníková, P., Birošová, L., Švorc, Ľ., et al. (2015). Newly synthesized indolizine derivatives – antimicrobial and antimutagenic properties. *Chemical Papers*, 69(7), pp. 983-992. 2015, IF 1,468.
3. Šaková, N., Olejníková, P., Kalináková, B., Pangallo, D., Lakatoš, B., Nagyová, K., Valachovičová, M., Birošová, L. Comparative study of adult Slovak vegetarians and meat-eaters gut microflora(2015) Journal of Food and Nutrition Research, 54 (3), pp. 260-269. IF:0,804.
4. Šimkovič, M., Olejníková, P., Mat'at'a, M., Žemla, P., Vilimová, V., Farkašová, L., Varečka, L. Nutrient transport into germinating Trichoderma atroviride conidia and development of its driving force(2015) Microbiology (United Kingdom), 161 (6), pp. 1240-1250. Cited 1 time.IF: 2,557 (5years IF: 3,026).
5. Kavala, M., Brezová, V., Švorc, Ľ., Vihonská, Z., Olejníková, P., Moncol, J., Kožíšek, J., Herich, P., Szolcsányi, P. Synthesis, physicochemical properties and antimicrobial activity of mono-/dinitroxyl amides (2014) Organic and Biomolecular Chemistry, 12 (25), pp. 4491-4502. IF: 3,562.
6. Mackulak, T., Prousek, J., Smolinská, M., Olejníková, P., Takáčová, A., Drtil, M.Degradation and toxicity changes in aqueous solutions of chloroacetic acids by Fenton-like treatment using zero-valent iron (2013) Chemical Papers, 67 (12), pp. 1594-1598. IP 1,193.

7. Takáčová, A., Smolinská, M., Olejníková, P., Mackuľak, T., Čík, G.A study of the phototoxic effect of Benzo[a] Pyrene on bacteria (2013) Central European Journal of Biology, 8 (11), pp. 1127-1134. IP 1,193.
8. Olejníková, P., Kurucová, M., Švorc, L., Marchalín, Š.Induction of resistance in Mycobacterium smegmatis (2013) Canadian Journal of Microbiology, 59 (2), pp. 126-129. IP 1,182.

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Chapter 5

ADVANCED GLYCATION END PRODUCTS IN *ESCHERICHIA COLI* K-12: A SIGN OF AGING

Rositsa Tsekovska[1], Yordan Handzhiyski[1], Elitsa Boteva[1], Kiril Kirilov[1], Toshimitsu Niwa[2], Ivan Ivanov[1] and Roumyana Mironova[1,*]

[1]Institute of Molecular Biology "Roumen Tsanev," Bulgarian Academy of Sciences, Sofia, Bulgaria
[2]Faculty of Health and Nutrition, Shubun University, Ichinomiya, Japan

ABSTRACT

Recent studies have challenged the paradigm that bacteria do not age and are immortal. Stewart et al. (PLoS Biology, 2005, 3(2), e45) provided an intriguing evidence that during division *Escherichia coli* K-12 exhibits functional asymmetry, leaving behind a mother cell with delayed growth and survival rate and a younger daughter cell, empowered to successfully perpetuate the species over time. In view of this new finding, bacteria, and in particular *E. coli*, emerged as a promising model for exploration of basic mechanisms of aging. It is not yet clear to what extent pro- and eukaryotic cells age similarly but at least some features of aging,

* Corresponding Author address; E-mail: rumym@bio21.bas.bg.

especially at the molecular level, should be common. Spontaneous chemical reactions including hydrolysis, oxidation and glycation (the Maillard reaction) are well known to deteriorate macromolecular structure and function. The Maillard reaction, yielding the so-called Advanced Glycation End Products (AGEs) on proteins, DNA and amino-lipids, has been long associated with diseases (diabetes, Alzheimer's and Parkinson's diseases) and aging in humans. We have demonstrated that despite the short life span of *E. coli* of tens of minutes to hours, its chromosomal DNA accumulates AGEs under normal growth conditions (Mironova et al., Mol. Microbiol., 2005, 55(6), p1801). Such progressive modification of a key biological molecule provides an independent line of evidence for *E. coli* aging. This article reviews data on the Maillard reaction as a cause of stochastic damage in *E. coli*, and subsequently, of *E. coli* aging.

Keywords: aging, *Escherichia coli*, Maillard reaction, glycation, AGEs

INTRODUCTION

"Aging" and "death" – everybody knows the meaning of these two words and scientific definitions can add little to that knowledge. What science is trying to do is to answer the questions "Why and how do we age and die" and "Is it possible to counteract these processes?" There is a plethora of aging hypotheses, and while some of them are contradicting, others are compatible and complimentary. Regarding the rather philosophic question "Why do we age?," the evolutionary theory of aging is thought to provide the most relevant explanation. The "mutation accumulation" (Medawar, 1952), the "antagonistic pleiotropy" (Williams, 1957) and the "disposable soma" (Kirkwood, 1977, 2005) are examples of hypotheses of aging that complement each other. Briefly, according to these hypotheses and in compliance with the Charles Darwin theory of evolution (Darwin, 1859), mutations, reproduction and natural selection are evolutionary tools elaborated to best adapt the life forms on our planet to dynamic changes. The life continuity among multicellular organisms is guaranteed by the clear discrimination between somatic and germ line cells. The mortal somatic cells (the soma) make up the most of the multicellular body, thereby guarding the remaining small portion represented by the germ cells. Only mutations in the germ cells can be passed on to the next generations and fixed. Mutations in the somatic cells are not under selection pressure and can accumulate in cells and tissues of adults thus contributing to aging and age-related diseases. Mutations that contribute also

to aging and death ensure generation continuity and may be the cost organisms have to pay in order to change and evolve (Saint-Ruf et al., 2014; Kram and Finkel, 2015). Experimental data reveal that the tradeoff against an extended lifespan is reproduction failure, as far as animals modified to live longer are often sterile (Friedman and Johnson, 1988; Leroi et al., 1994; Henderson and Johnson, 2001; Bartke and Chandrashekar, 2003). If we now extrapolate longevity to immortality this should result in an eternal life that does not reproduce and change. Such a static and uniform life does not fit in the dynamic abiotic picture of our planet and is obviously no more than a science fiction.

Bacteria are single-celled organisms reproducing by binary fission and most of them do not show visible signs of differentiation between the two fission progeny. The intestinal bacterium *Escherichia coli* is well known to experience the so-called "conditional senescence" upon nutrient deprivation and starvation (Nyström, 1999, 2003). The more intriguing question, however, is "If there were no nutrient restriction (no starvation) and no invaders (phages, antibiotics) in the environment, would bacteria live forever? It is obvious that higher eukaryotes including humans do age and die despite no food limitation, and even more, life expectancy appears to be inversely related to the caloric intake (Colman et al., 2014). Does the same hold true for bacteria as well? One decade ago the answer to this question appeared negative. Bacteria, including *E. coli* reproducing *via* binary fission, resemble the immortal germ line cells of multicellular organisms and it was believed that they do not exhibit an instant (replicative) aging (Nyström, 2002).

A prerequisite for replicative aging of unicellular organisms is the asymmetric division. An example of such a division mode is the lower eukaryote *Saccharomyces cerevisiae*, which reproduces by budding (Figure 1A). The term "budding" refers to the fact that one of the fission products is smaller and appears to be the progeny (the daughter) cell of the bigger (mother) cell that behaves like a parent giving birth (budding) to the daughter cell. More than half a century ago, it became clear that the yeast mother cells undergo replicative aging and thereafter die (Barton, 1950; Mortimer and Johnston, 1959). Not all asymmetrically dividing single-celled organisms, however, demonstrate such a clear distinction between the two fission products. For example, the mother cell of the yeast *Schizosaccharomyces pombe* (Figure 1B) is visibly enlarged but the size difference between the two progeny cells is quite small for the process to be called "budding" (Barker and Walmsley, 1999). The asymmetrically dividing *Caulobacter crescentus* (Figure 1C) was the first bacterium shown to exhibit features of replicative

aging (Ackermann et al., 2003). The asymmetric division tells us that even among unicellular organisms there are primary signs of differentiation and development. What is the difference between the two asymmetric products – the mother and the daughter? The yeast mother cell, for example, takes on itself most of the damaged cellular components (Nyström and Liu, 2014; Higuchi-Sanabria et al., 2014; Hill et al., 2016), and in that way, it resembles the mortal altruistic soma of multicellular species. The tradeoff is a juvenile daughter cell with enhanced odds of survival, which in turn becomes a mother, giving rise to the next generation. The asymmetric division is an evolutionary solution to avoid toxic chemical buildup. As John Baynes elaborated (Baynes, 2000): "In the continuing struggle between chemistry and biology, chemistry is always the short-term, tactical winner – death of the individual is inevitable. However, barring the extinction of species, biology is the long-term, strategic victor – life survives, and the struggle continues."

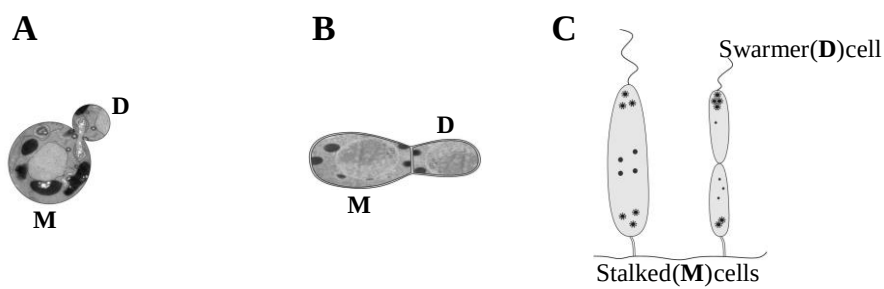

Figure 1. Asymmetric division of unicellular organisms. (A) *Saccharomyces cerevisiae;* (B) *Schizosaccharomyces pombe;* (C) *Caulobacter crescentus;* M-mother cell; D-daughter cell.

REPLICATIVE AGING OF *E. COLI*

When observed under the microscope, *E. coli* appears to divide symmetrically by producing two identical daughter cells (Figure 2) and it has been accepted *a priori* that this bacterium is *de facto* immortal (Rose, 1991). In 2005, Stewart et al. challenged this notion by suggesting that there may be a functional asymmetry between the two seemingly equal daughter cells. The authors were motivated by the observation that some cellular components with relatively long half-life and small diffusion rate accumulate predominantly at one of the two poles of the new-born bacterial cell. This is invariably the pole

inherited from the parent cell away from the division septum, which becomes the new pole (Figure 2). Stewart et al. (2005) observed the division of 94 single cells as they form colonies on flat surface in real time *via* fluorescence microscopy up to the ninth generation (nearly 500 progeny cells *per* colony). They were able to measure the cell size and the time each cell needed to divide and to calculate the growth rate of each individual cell. The basic conclusion was that in contrast to the stable growth rate of the new pole cells, that of the old pole cells progressively slows down, but neither the size of the new pole cell nor the time it needs to resume division are indicative of the presence of a juvenile phase in *E. coli*. In addition, the old pole cells produced less offspring biomass and deaths among them were more frequent. To explain these observations, the authors suggested that there should be a functional asymmetry between the two fission products due to differential turnover and preferable accumulation of some cellular components in the cell poles.

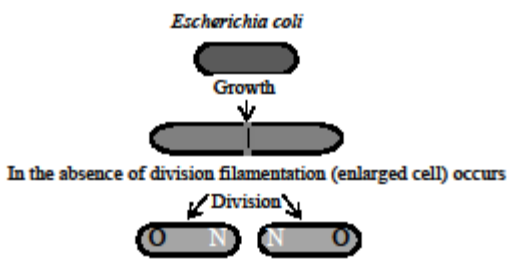

Figure 2. Symmetric division of E. coli. There is no morphological difference between the two fission products. O – old pole; N - new pole.

The finding of Stewart and co-workers was questioned by the study of Wang et al., (2010), who failed to find any difference in the growth rate between the old and new pole *E. coli* cells over hundreds of generations. Here, it is important to note that i) the experimental setup of the two studies differed significantly, and ii) while demonstrating robust *E. coli* growth over many generations, the study of Wang et al. (2010) did not argue against *E. coli* aging (Rang et al., 2011). Wang and colleagues used the so called "mother machine" representing a high-throughput microfluidic liquid-culture devise ensuring long-term delivery of fresh medium to each cell and measuring cell parameters such as length and generation time over hundreds of generations. Thus by observing more than 10^5 individual cells for 200 generations, the authors did not find any cumulative decrease in the growth rate of the old pole (mother)

cells as compared to that of the new pole (daughter) cells. The interpretation of this result was that there is no "built-in growth-based aging mechanism in *E. coli.*"

Interesting observations came from the filamentation of the bacterial cells. The filamentation phenotype (enlarged cells) results from cell growth that is not followed by division. While the filamentation rate of the daughter cells remained constant over time (~ 1 filamentation per 100 generations), there was more than 10 fold increase in the filamentation rate of the mother cells with advancing replicative age. First filamentaions of the mother cells occurred after around 50 divisions while next filamentation intervals declined with time following a power law distribution, which points to a link between filamentation and aging, or more likely to common roots of these two phenomena. Because filamentation is a hallmark of the SOS response (global response to DNA damage), this means that the mother cells, inheriting the same old pole over many generations, switch on more often the SOS alarm perhaps due to DNA damage. This suggestion was confirmed by the increased mortality rate of an isogenic *E. coli* strain with blocked SOS response. Additionally, there was a correlation between the filamentation and death rates of the wild type strain with functional SOS response (compare Figures 3C and 4B in the study of Wang et al., 2010).

What is the cause of aging and death of individual cells in the otherwise expanding bacterial population? Wang et al. (2010) hypothesized that "the mother cell must inherit an unknown "factor" that serves as a long-term memory from one generation to the next and causes filamentation" and death. The unknown death-causing factor might be asymmetrically distributed aggregates in *E. coli* (Lindner et al., 2008; Winkler et al., 2010) and/or metabolic slowdown at the old pole cell wall (den Blaauwen et al., 2008). Reasonably, because of the high death rate, the authors excluded stochastic fluctuations in DNA damage or metabolism as a cause of the observed mortality of the wild type *E. coli* strain. However, accumulating (i.e., age-dependent) DNA damage should not be excluded as a probable death-causing factor. We are tempted to speculate that the robust *E. coli* growth could be in part contributed by the diploid and tetraploid state of actively dividing *E. coli* (generation time ~ 20 minutes). Under such conditions, bacterial cells are less susceptible to DNA damage, which therefore can accumulate and become physiologically evident in aging cells at later growth stages.

In the bacterial world, immortality would mean that each cell divides and never dies assuming that division is not death and excluding accidents (stochastic deaths, e.g., due to mutations). Is this the case for *E. coli*? In

natural habitats, *E. coli* rarely exhibits robust growth for long periods and "feast and famine" is its usual life style. Thus, the "mother machine" of Wang et al. (2010) provides an excellent model, showing what would happen if *E. coli* were permanently living in nutrient-rich and sterile environment. What we see is that under such conditions the bacterial population is continuously expanding at the expense of aging and death of individual cells. The bacterial population survives over time in the same way, in which higher organisms die but species continue to live. Thus, the study of Wang et al. (2010) helped debunk the myth of bacterial immortality.

CONDITIONAL SENESCENCE OF *E. COLI* – THE FIVE PHASES OF BATCH GROWTH

In the laboratory, *E. coli* is usually batch cultured and under such conditions its life cycle is composed of five phases as outlined by Finkel (2006) (Figure 3). When transferred to fresh LB medium at low density *E. coli* experiences a latent period (*lag phase*) of several hours, needed to adjust metabolically to the new environment before entering the active reproductive (*exponential* or *logarithmic*) *phase*. Among all growth phases, the exponential phase most closely resembles the robust growth in the study of Wang et al. (2010) given that fresh medium is continuously flowing through the culture. During this reproductive phase, *E. coli* divides rapidly (each ~20 min in rich media) over several hours. Using an optical trapping methodology, Ericsson et al. (2000) did not find evidence for the presence of dead cells among exponentially growing *E. coli* cells at 0.1% limit of detection. However, another study (Gallant and Palmer, 1979) reported that 0.5% of exponential *E. coli* cells failed to form colonies on solid agar. Considering that such mortality rate is much higher than spontaneous mutation rate ($\sim 10^{-7}$), it seems likely that during logarithmic phase in batch cultures, *E. coli* undergoes replicative aging and death in the same fashion as in the "mother machine" of Wang et al. (2010).

After exponential growth, batch cultured *E. coli* enters a *stationary phase* characterized by no obvious increase or decrease in the total number of cells. This phase lasts 2 to 3 days, during which most cells steadily loose replicative capacity but remain intact for extended periods of time (Ericsson et al., 2000). Thereafter follows the onset of the fourth, *death phase*, ending up with vigorous decay of the bacterial population. In this phase, up to 99% of the cells

lyse and die, thereby releasing a large amount of cell debris. It is prodigious that even in the absence of nutrients life wins over death as far as a small fraction (1%) of *E. coli* cells survives at the expense of the dead siblings. In favor of the "disposable soma" hypothesis of aging (Kirkwood, 1977), this is the second example of an altruistic behavior of *E. coli*. In the "mother machine" of Wang et al. (2010), each one of hundreds to thousands mother cells dies, so that daughter cells can survive. In the death phase of batch-cultured *E. coli* the self-sacrifice is even greater, because almost all cells die giving chance to the minor rest of cells to escape death and adapt to the new hostile environment.

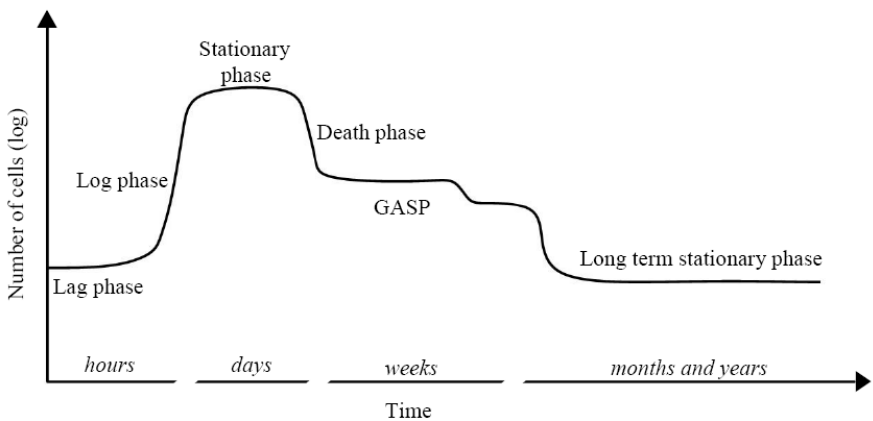

Figure 3. The five phases of *E. coli* growth in batch cultures. GASP – "Growth Advantage in Stationary Phase" phenotype.

In the last growth phase called *long-term stationary phase* (LTSF), commencing in *ca*. one week old batch cultures, some survivors of the death phase acquire the so called "growth advantage in stationary phase" (GASP) phenotype (Zambrano and Kolter, 1996; Vulic and Kolter, 2001). GASP means that if such cells are mixed with younger cells (e.g., from 1 day old cultures) they will displace them after prolonged (7-10 days) co-culturing (Finkel, 2006). It is remarkable that such cells may retain viability up to 10 years in liquid cultures without the addition of nutrients (Gagliardi et al., 2016). GASP is not a physiological phenomenon but a genetically determined phenotype. High throughput proteomics and microarray studies as those published recently (Arunasri et al., 2014; Gagliardi et al., 2016) are expected to provide insight into the heterogeneous genetic nature of this intriguing phenotype. Common feature of all GASP mutants is their increased ability to

catabolize one or more amino acids as the only energy source (Zinser and Kolter, 1999, 2000, 2004; Zinser et al., 2003). Interestingly, among GASP mutations, there are mutations in the *rpoS* gene attenuating the expression of the stress response regulator RpoS (σ^S) in *E. coli* (Zambrano et al., 1993). In opposite, *E. coli* mutants in the *rssB* gene (promoting photolytic RpoS degradation) express a more stable RpoS and exhibit reduced mortality. However, such cells show reduced growth rate and fitness as compared to the wild type ancestor (Fontanie et al., 2008). This latter observation supports the "antagonistic pleiotropy" hypothesis of aging (Williams, 1957) claiming that there is a negative feedback between reproductive success and longevity. In the case with *E. coli*, the tradeoff against the decreased mortality is reduced growth rate and compromised adaptivity.

AGING AT MOLECULAR LEVEL – THE GLYCATION HYPOTHESIS OF AGING

Studies with *E. coli* provided firm evidence that explicit asymmetric division and juvenile phase are not mandatory for cellular aging and that the basic principles of aging have to be searched at molecular level. Now, we have to face those theories of aging that address the question "How do we age?" Fortunately, these theories are not mutually exclusive but rather converging to a finite number of aging mechanisms. The "genome maintenance" hypothesis of aging (Alexander, 1967; Karanjawala and Lieber, 2004), for example, coincides well with the "oxidative damage/free radical" (Harman, 1956) and the "Maillard reaction" (Cerami, 1986; Monnier, 1989) hypotheses of aging. According to the "genome maintenance" hypothesis, deterioration of DNA repair and accumulating DNA damage in somatic cells of multicellular organisms are crucial for aging. On the other side, the other two hypotheses point to some causes of DNA damage such as oxidation ("oxidative damage/ free radical" hypothesis) and glycation (the "Maillard reaction" hypothesis). In turn, from a chemical point of view oxidation and glycation are tightly interrelated molecular events often designated as glycoxidation.

The Maillard reaction is named after the French chemist Louis Camille Maillard, who was first to show that glucose reacts with amino acids *in vitro* (Maillard, 1912). In the early stage of this reaction, the NH_2-groups of primary amines react spontaneously (no catalysis) and reversibly with the carbonyl groups of glucose and other carbonyl compounds to form a Schiff base

(aldimine). Then, at neutral pH, the Schiff base undergoes an intramolecular rearrangement to a more stable Amadori product (ketoamine) (Hodge, 1955). In the late stage of the Maillard reaction, because of additional chemical transformations, the Amadori products give rise to the so-called Advanced Glycation End Products (AGEs) (Figure 4).

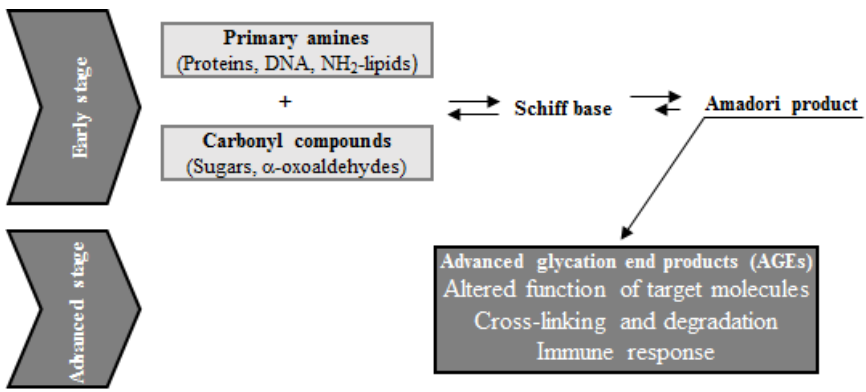

Figure 4. The Maillard Reaction.

AGEs are an extremely heterogeneous group of covalent adducts and the chemical structure of a number of AGEs has been identified so far (Nakayama et al., 1980; Pongor et al., 1984; Ahmed et al., 1986; Sell and Monnier, 1989; Ahmed and Thornalley, 2003; Thornalley et al., 2003; Thorpe and Baynes, 2003; Lapolla et al., 2013). Some AGEs form intra- and intermolecular cross-links and may cause covalent aggregation of affected macromolecules. Figure 5 shows the chemical structure of some AGEs with cross-linking properties, promoting protein-protein interactions and aggregation.

The term "Maillard Reaction Products" (MRPs) refers to both Amadori products and AGEs. Studies in the late 1960s showed that the Maillard reaction does take place in the human body as well, where it accounts for the formation of a glucose-derived Amadori product at the N-terminus of the hemoglobin β-chain (Rabhar, 1968, 2005). Glycated hemoglobin (HbA_{1c}) increases with aging of the red blood cells of healthy subjects and to a higher degree in erythrocytes of diabetic patients. Because of that, HbA_{1c} is nowadays a routine hyperglycemic marker. In the mid of 1980s, it has been hypothesized that glycation plays an important role in the pathogenesis of diabetic complications and aging (Cerami, 1986; Monnier, 1989). Subsequently, this hypothesis found a lot of experimental support, and the link between

glycation, diabetes and aging has been hotly debated in many reviews (Brownlee, 1995; Thorpe and Baynes, 1996; Ulrich and Cerami, 2001; Nass et al., 2007; Hellwig and Henle, 2014; Sadowska-Bartosz and Bartosz, 2016).

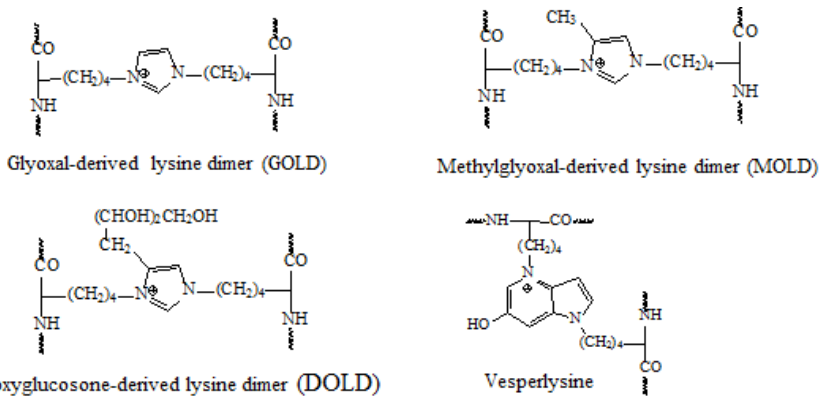

Figure 5. Lysine-lysine AGEs cross-links.

Protein cross-linking is only one of the many effects of the Maillard reaction and in fact, Johan Bjorksten postulated the role of cross-linking in aging (Bjorksten, 1958; Bjorksten and Tenhu, 1990) many years before the realization that the Maillard reaction proceeds in the human body. Many age-related disorders are associated with AGEs-promoted protein cross-linking (Ajith and Vinodkumar, 2016; Rabbani et al., 2016). Cross-linking of skin collagen is responsible for wrinkling and other age related dermal changes (skin dryness, appearance of spots, and decrease in elasticity) (Verzijl et al., 2000; Pageon, 2010). Cross-linking of lens proteins contributes to age-related cataract formation (Linetsky et al., 2008; Nagaraj et al., 2012; Pescosolido et al., 2016). AGEs take part also in the formation of atherosclerotic plaques in the arterial wall, which in case of rupture provoke cardiovascular events (Hanssen et al., 2014; de Vos et al., 2016). AGEs are located at the periphery of Lewy bodies in patients with Parkinson's disease (Castellani et al., 1996; Münch et al., 2000). In Alzheimer's disease, glycation of amyloid β-peptide exacerbates its toxicity and contributes to neurodegeneration (Li et al., 2013; Jana et al., 2016; Vicente Miranda et al., 2016). The Maillard reaction is implicated in aging not only through protein glycation. AGEs and perhaps AGEs cross-links appear also on DNA (see below), and in 2002 John Baynes was appealing that, it is "time to focus on DNA" (Baynes, 2000, 2002). Old people and diabetic patients are at higher risk of developing cancer and Lee

and Chan (2015) suggested that DNA damage including DNA-AGEs could be a biological link between diabetes and cancer.

MAILLARD REACTION AND REPLICATIVE AGING OF *E. COLI*

Despite the many data, demonstrating the role of the Maillard reaction in human aging and age-related diseases, its implication in *E. coli* metabolism remained unexplored for a long time. Model studies focused mainly on the physiological significance of MRPs present in the culture media (Helou et al., 2014) but did not raise the question of whether glycation occurs within *E. coli* cells. As a spontaneous (non-enzyme catalyzed) reaction, the Maillard reaction proceeds slowly under physiological conditions (pH ~7, 37°C) and there was a silent agreement that this reaction is unlikely to occur in bacteria. For example, the formation of Schiff bases requires 1 - 2 hours to reach equilibrium in the absence of a catalyst, while the generation time of a well-nourished *E. coli* is only 20 minutes. We went on to reconsider this assumption when searching for an explanation for the striking behavior of a recombinant human interferon-gamma (IFNγ), expressed in *E. coli*. Despite the lack of cysteine residues, the protein underwent progressive covalent aggregation during storage under both aerobic and anaerobic conditions, which excluded oxidation (i.e., formation of disulfide bridges and tyrosine dimers) as the one and only cause of the observed phenomenon. Covalent oligomerization *via* cross-linking AGEs seemed a reasonable explanation of the covalent IFNγ aggregation under oxygen-free conditions. We performed relevant investigations and found that in addition to IFNγ (Mironova et al., 2003), endogenous *E. coli* proteins (Mironova et al., 2001) and chromosomal DNA (Mironova et al., 2005) are involved in glycation under normal growth conditions.

Does the Maillard reaction contribute to replicative aging and conditional senescence in *E. coli*? The studies on glycation in *E. coli* have a relatively young history and since our first publication in 2001 (Mironova et al., 2001), roughly 70 articles appeared on that topic. In spite of the limited number of studies, however, some glycation signatures in the *E. coli* aging mechanism are already recognizable. The pioneer studies of Stewart et al. (2005) and Wang et al. (2010) linked the aging strategy of *E. coli* to the asymmetric distribution of some cellular components including protein aggregates. Next investigations indeed showed that heat shock as well as overexpression of

foreign proteins in *E. coli* lead to the accumulation of protein aggregates in one of the cell poles (Lindner et al., 2008; Winkler et al., 2010). Such unequal partitioning allows for asymmetric inheritance of damaged proteins during cell division and results in higher growth rates of damage-free daughter cells. Data also indicate that polar aggregation of cytosolic proteins is mainly driven by nucleoid occlusion rather than by an active, genetically encoded mechanism (Winkler et al., 2010). Aggregation of native proteins in *E. coli* is promoted by stress conditions (oxidative stress, heat shock), as well as by errors during transcription and translation, resulting in aggregation even in the absence of an external stress (Mogk et al., 2003).

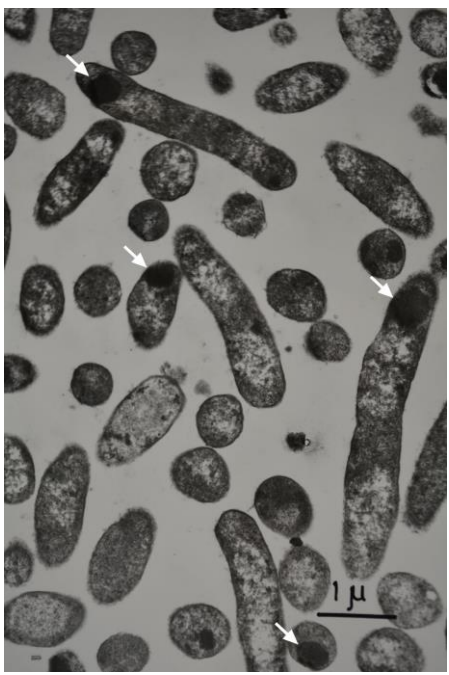

Figure 6. Electron micrograph of *E. coli* cells overexpressing IFNγ. White arrows indicate inclusion bodies with polar (membrane) localization.

The contribution of glycation-mediated cross-linking to protein aggregation in *E. coli* is poorly studied. We have previously shown that while expressed in *E. coli*, human IFNγ accumulates both Amadori products and AGEs, depending on the host strain and fermentation conditions. We also observed that some internal amino acid residues (most likely Arg and Lys) are involved in IFNγ cross-linking and covalent dimerization (Mironova et al.,

2003; Boyanova et al., 2008). The protein was abundantly expressed (up to 50% of the total protein) and was sequestered by the bacterial cells in insoluble inclusion bodies with polar localization (Tsanev and Ivanov, 2002) (Figure 6).

We suggest that AGEs-promoted cross-linking may be a factor contributing to the observed aggregation of the overexpressed recombinant IFNγ. Most studies focus on the presence specifically of protein aggregates in *E. coli* inclusion bodies, however, we have recently observed that IFNγ inclusion bodies can contain nucleic acids as well (plasmid DNA and rRNA) (Ahmed et al., 2011; Krachmarova et al., 2011, Popov, 2012) (Figure 7). As shown in Figure 8, when glycated *in vitro*, *E. coli* DNA gains in molecular mass, which is indicative of DNA cross-linking. This result, together with our observation that *E. coli* chromosomal DNA accumulates AGEs (Mironova et al., 2005), implies that DNA cross-linking and aggregation are likely to occur also *in vivo*. In addition, AGEs-crisscrosses between DNA, proteins and other primary amines might contribute to an even more complex structure of the *E. coli* inclusion bodies.

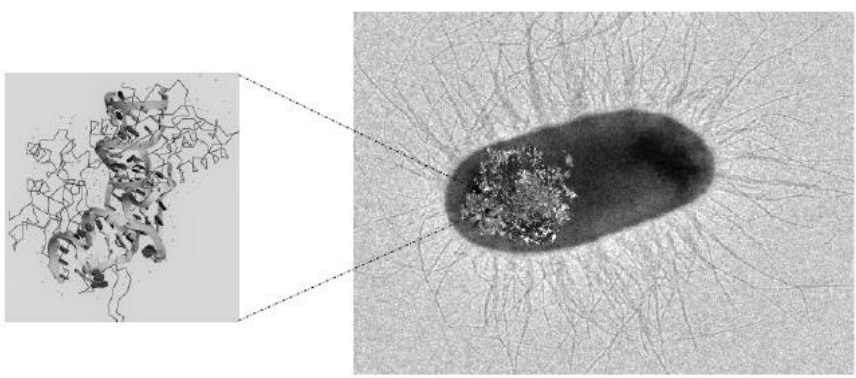

Figure 7. *E. coli* inclusion bodies contain both proteins and nucleic acids.

Yet another observation of our group supports the *E. coli* aging mechanism proposed by Stewart et al. (2005) and Wang et al. (2010). To express human IFNγ in *E. coli*, we have cloned the IFNγ gene in a multicopy plasmid under a strong (phage) constitutive promoter. With advancing fermentation of *E. coli* cells, we observed a phenomenon called plasmid segregation. With time, some cells lost their plasmids, began to grow faster and displaced the plasmid-bearing cells, which resulted in reduced IFNγ yield.

To explain this observation, we proposed a model based on the coupled transcription and translation in *E. coli*. Because *E. coli* lacks a nuclear envelope, transcription takes place in the cytosol, where translation of the commencing mRNA starts before transcription is terminated. Assuming that IFNγ aggregation and inclusion bodies formation may occur while IFNγ is still being synthesized, our model predicts that the whole complex, including plasmid DNA, mRNA and the resulting IFNγ together with the supporting ribosomes (polysomes), might be tethered by the inclusion bodies to the cell poles and anchored into the cell membrane (Popov et al., 2011). This model explains why plasmid DNA and rRNA are present in the inclusion bodies and how plasmid segregation may occur. In fact, plasmid segregation seems to be tightly linked to the *E. coli* aging mechanism. The recombinant plasmids and the encoded protein impose a metabolic burden on *E. coli* cells and are therefore partitioned with other surplus components in mother cells (in terms of Stewart et al. (2005)), resulting in relieved growth of the daughter cells.

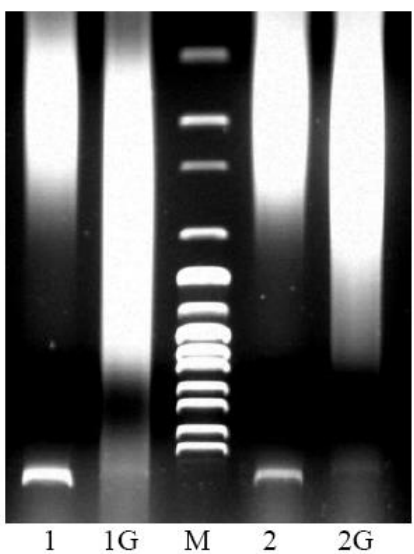

Figure 8. Glycation-induced increase in the molecular mass of *E. coli* chromosomal DNA. DNA was isolated from *E. coli* strains 33W1485 (1) and AB1157 (2) and sonicated to an average length of 1000 bp. Samples 1G and 2G were incubated for two weeks in 0.1 M glucose 6-phosphate at 37°C under sterile conditions. Analysis was performed on 0.7% agarose gel. M - 1 kb DNA Ladder.

MAILLARD REACTION AND CONDITIONAL SENESCENCE OF *E. COLI*

Stewart et al. (2005) followed the division of single cells after plating onto solid agarose and observed a delayed growth of the old pole (mother) cell in contrast to Wang et al. (2010), who reported on a stable growth of both mother and daughter cells during continuous fermentation in liquid culture. We suggest that due to overgrowth and accompanying quorum sensing (Miller and Bassler, 2001) on plane surface, Stewart et al. (2005) observed phenomena close to conditional senescence rather than a true replicative ageing, which may explain the discrepancy between the two studies. Using optical tweezers, Ericsson et al. (2000) made the interesting observation that only one of the two daughter cells of growth-arrested (stationary) *E. coli* that appear after re-nutrition "was able to divide a second time, indicating that localized cell deterioration, inherited by only one of the daughters, may occur." This data imply that common events may underlie both conditional and replicative aging of *E. coli*. In fact, the stationary phase and especially LTSP are not static, because the apparently constant cell density results from dynamic equilibrium between dying and newly emerging cells. In other words, the aging bacterial population does not loose proliferative function. It is disputable yet whether *E. coli* entry into stationary phase and senescence are programmed phenomena, and in a detailed review on that topic, Nyström (2003) concluded that bacterial senescence is linked to stochastic deterioration, rather than programmed death pathways, and self-inflicted oxidative damage may be a causal factor in age-related deterioration of both prokaryotes and higher organisms. The spontaneous (not genetically encoded) Maillard reaction may cause such a stochastic deterioration of life essential amines including proteins, DNA and amino-lipids and thus be an important player in the major events of *E. coli* senescence, which are mutability, death and adaptation.

We have previously shown that the severity of the carbonyl stress in *E. coli*, as mirrored by the level of AGEs in the total protein, depends on the culture medium composition (Dimitrova et al., 2004). Subsequent independent studies have confirmed this observation (Pepper et al., 2010; Kram and Finkel, 2015). The presence of reducing sugars such as glucose and fructose in the LB medium at concentrations above 0.2% caused reduced viability of cells in stationary phase and accelerated death in the death phase, which correlated positively with the accumulation of CML (AGEs) in the total protein. In addition, the supplementation of LB medium with 2 mM methylglyoxal (MG)

lowered dramatically (million times) the viability of stationary cells (Pepper et al., 2010). When LB was replaced with richer media containing higher concentrations of Yeast extract and Tryptone (Soytone), it was realized that the rich the medium the severe is the death phase and the less the degree of recovery in the LTSP (Kram and Finkel, 2015). High levels of CML in the total protein accompanied the onset of the death phase for all media tested, and the severity of the death phase correlated positively with the spontaneous mutation rate to rifampicin resistance (rif^r) in late exponential cells. In these cells, the amount of AGEs-modified proteins depended also on the culture volume with bigger volumes promoting higher glycation levels (Kram and Finkel, 2014). In this latter study, enhanced glycation did not always correlate with increased oxidative stress (evaluated by OxyR induction), which is not striking, bearing in mind that glycation may happen also under anaerobic conditions.

Kram and Finkel (2014, 2015) demonstrated that the accumulation of AGEs in endogenous proteins is a hallmark of *E. coli* senescence. Importantly, there was a positive correlation between the level of protein-bound AGEs and the frequency of spontaneous mutations in stationary cells. This correlation indirectly indicates that glycation of DNA might also be a factor in bacterial senescence. That DNA may react with reducing sugars *in vitro* has been shown (Bucala et al., 1984) but it is a discovery of the last decade that reducing sugars may react with DNA in living organisms (Mironova et al., 2005; Fleming et al., 2007). By adding glycation inhibitors (aminoguanidine (AG), aspirin (ASA), pyridoxal 5'-phosphate (PLP), pyridoxamine (PM), pyridoxin (PN) and thiamine (B1)) to the LB medium, we have shown that the amount of Amadori products (AGEs-precursors) in *E. coli* chromosomal DNA correlates positively with the mutation frequency to rif^r phenotype in both exponential and stationary (3 days old) cells (Mironova et al., 2009; Handzhiyski et al., 2012) (Figure 9).

Based on unpublished observations, Kram and Finkel (2015) made the interesting notion that "high mutation frequency, in and of itself, does not necessarily lead to a more severe death phase since *mutS*, *mutL*, or *dam* strains, with mutation frequencies up to 100 times that of wild-type cells, can survive long-term stationary-phase incubation when grown in batch culture." We cite this notion to stress on the fact that mutagenic factors including glycation are the culprit causing death but also the genetic/phenotypic diversity necessary for bacterial survival and adaptation.

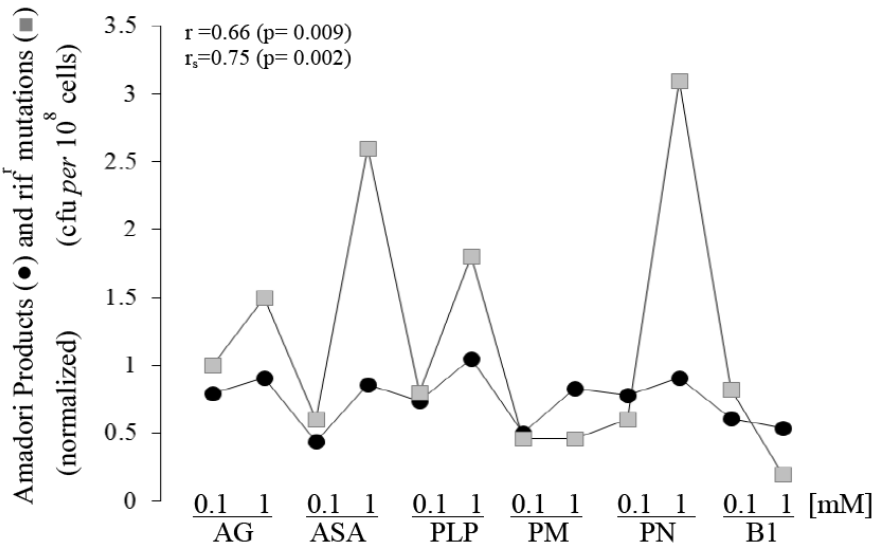

Figure 9. Early glycation and spontaneous mutagenesis in *E. coli*. Correlation between the amount of Amadori products (●) in the chromosomal DNA and the spontaneous mutation frequency to rif^r (■) of *E. coli* stationary (3 day old) cells. Data for Amadori products are normalized, while the mutation frequency is expressed as median cfu *per* 10^8 cells of 10 independent cultures tested. Shown are the Pearson (r) and the Spearman (r_s) correlation coefficients.

DEFENSE AGAINST GLYCATIOIN AS AN ANTI-AGING STRATEGY

Experiments have shown that glycation levels and spontaneous mutation rate in *E. coli* are manageable. In the study of Kram and Finkel (2014), the anti-glycation agent carnosine exhibited pronounced inhibitory effect on the death phase, restoring the viable cell counts up to five orders of magnitude. Other compounds found in this study to protect against high concentrations of glucose and MG were aminoguanidine and folic acid, while aspirin and grape seed extract abrogated the effect of MG but not that of glucose (Pepper, 2007). In natural habitats, *E. coli* cannot rely on the delivery of such compounds and has elaborated diverse mechanisms for anti-glycation defense. These mechanisms operate at all steps of the Maillard reaction starting with detoxification of carbonyl compounds, going thorough degradation of Amadori products, and ending up with secretion of AGEs.

One of the best-studied toxic carbonyl compounds in *E. coli* is MG (Kosmachevskaya et al., 2015). MG is produced in *E. coli in vivo* either spontaneously (Ahmed et al., 1997) or enzymatically (Hopper and Cooper, 1972; Tötemeyer et al., 1998) and if not detoxified its accumulation ultimately causes cell death (Freedberg et al., 1971; Rekarte et al., 1973; Ackerman et al., 1974; Kadner et al., 1992). The main route for MG detoxification is the glyoxalase I/II system (MacClean et al., 1998; Ferguson, 1999), and some data point to the presence of an additional glyoxalase III in *E. coli* (Misra et al., 1995; Subedi et al., 2011). Other enzymes involved in MG detoxification are aldose reductases (Misra et al., 1996; Saikusa et al., 1987) and enzymes converting MG to acetol (encoded by the *E. coli* genes *yghZ*, *yafB*, *yqhE*, and *yeaE*) (Ko et al., 2005). Two independent studies published this year (Abdallah et al., 2016; Lee et al., 2016) reveal another group of enzymes in *E. coli* with glyoxalase/deglycase activity. These enzymes, HchA, YajL, YhbO and ElbB, share similarity with the human DJ-1 superfamily of proteins. When overexpressed in *E. coli*, the four DJ-1 homologs reduce the glyoxal (GO) dependent increase in intracellular AGEs. The apparent glyoxalase activity of YajL and YhbO seems to reflect their deglycase activity as far as these two enzymes are capable to repair GO- and MG-glycated proteins (Abdallah et al., 2016). The sequence similarity of the four *E. coli* proteins with human DJ-1, known to be associated with Parkinson's disease, and their expression primarily during *E. coli* stationary phase (Lee et al., 2016), point to the existence of common mechanisms for defense against carbonyl stress and senescence from bacteria to humans.

The removal of fructosamines (Amadori products), formed by glucose on human hemoglobin, is carried out by the enzyme fructosamine 3-kinese, acting as a deglycase (Delpierre et al., 2000; Delpierre et al., 2002). This enzyme phosphorylates the fructose residue at the third carbon atom, thus promoting the spontaneous breakdown of the bond between the protein and the reducing sugar. Fructosamine 3-kinese analog was not found in *E. coli* (Gemayel et al., 2007), where the decomposition of Amadori products was shown to result from the cooperative action of two enzymes – kinase (FrlD) and deglycase (FrlB) (Wiame et al., 2002). These enzymes utilize as a substrate Amadori products bound to free lysine residues but not to proteins. Therefore, they do not repair glycated proteins but rather sustain bacterial growth on fructoselysine, released during digestion of glycated proteins in the human intestine. Another *E. coli* enzyme, the Gcp glycopeptidase, has been suggested to directly repair Amadori products-modified proteins (AMPs), because: i) Gcp binds glycated proteins, ii) its depletion results in accumulation of AMPs,

and iii) the severe phenotype of Gcp depletion can be relieved under conditions of low intracellular glycation (Katz et al., 2010). Of note, Gcp is a conserved enzyme encoded by nearly every sequenced genome in all three domains of life, suggesting a universal involvement of Gcp in cellular aging. By analogy to higher eukaryotes, *E. coli* seems to have developed mechanisms for active secretion of AGEs outside cells in the form of free AGEs (bound to amino acids) and peptide-AGEs. It has been shown that low-molecular weight AGEs originate from high-molecular weight AGEs by proteolytic degradation, not carried out by the major ATP-dependent proteases but by an alternative metal-dependent proteolysis (Cohen-Or et al., 2011, Cohen-Or et al., 2013).

The mechanisms for repair of glycation damaged DNA in *E. coli* are scarcely studied. One of the major stable AGEs-adduct, resulting from the interaction of DNA with MG, is N(2)-(1-carboxyethyl)-2'-deoxyguanosine (N(2)-CEdG). Studies of Yuan et al., (2008) revealed that N(2)-CEdG is weakly mutagenic to *E. coli* and polymerase IV (pol IV), belonging to the Y-family of DNA polymerases, is the major enzyme responsible for bypassing the lesion *in vivo*. Steady-state kinetic measurements showed that nucleotide insertion, catalyzed by *E. coli* pol IV or its human counterpart (i.e., polymerase kappa), opposite the N(2)-CEdG lesion is both accurate and efficient. It is worth mentioning that the Y-family of DNA polymerases is highly conserved among all domains of life. Regarding repair of DNA modified with Amadori products, we have recently observed that *E. coli* lysates are capable of catalyzing the removal of fructosamine 6-phosphate from DNA (unpublished results) and studies are now in progress to identify the enzyme(s) responsible for this activity.

CONCLUSION

Current high-throughput live cell imaging helped debunk the myth of bacterial immortality, which was based on the observation that most bacteria divide symmetrically. During continuous exponential growth, the death rate of *E. coli* is orders of magnitude higher than the frequency of spontaneous mutations, which means that such deaths are unlikely to result from stochastic events. The studies of Stewart et al. (2005) and Wang et al. (2010) provided evidence that non-stochastic deaths amon proliferating cells are the outcome of an ongoing aging process in *E. coli*. The signs of aging were recognized in the functional asymmetry between the two *E. coli* fission progeny. The cells inheriting for many generations the old pole (mother cells) lost replicative and

survival potential over time in contrast to the new pole (daughter) cells. This intriguing finding raised the question about the reasons for metabolic deterioration of the old pole mother cells. The prevailing view among scientists is that stochastic deterioration, rather than programmed pathways account for aging of both pro- and eukaryotes. The Maillard reaction (glycation) and its advanced products (AGEs) have been long ago associated with aging and age-related human diseases. It was relatively recently, when we observed that this reaction takes place also in *E. coli* (Mironova et al., 2001), and therefore its implication in bacterial aging is still obscure. Accumulating experimental evidence indicates that glycation may play a dual role in *E. coli* physiology. Through promoting cross-linking and aggregation of proteins, DNA and other amines, the Maillard reaction may contribute to aging and death of proliferating *E. coil* cells (replicative aging) as well as to conditional senescence of stationary phase cells. However, through DNA damage, followed by error-pone repair, glycation may also contribute to enhanced mutagenesis and the appearance of CASP phenotype in LTSP cells, thus favoring bacterial survival and evolution. Finally, the pathways for defense against glycation are highly conserved and it appears that common molecular mechanisms underlie aging in all domains of life, from bacteria to humans, which gives us hope that *E. coli* may provide and excellent platform for deciphering the intimate mechanisms of aging.

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REFERENCES

Abdallah, J; Mihoub, M; Gautier, V; Richarme G. The DJ-1 superfamily members YhbO and YajL from Escherichia coli repair proteins from glycation by methylglyoxal and glyoxal. *Biochem Biophys Res Commun*, 2016 Feb 5;470(2):282-286.

Ackerman, RS; Cozzarelli, NR; Epstein, W. Accumulation of toxic concentrations of methylglyoxal by wild-type Escherichia coli K12. *J Bacteriol*, 1974 Aug;119(2):357–362.

Ackermann, M; Stearns, SC; Jenal, U. Senescence in a bacterium with asymmetric division. *Science*, 2003 Jun 20;300(5627):1920.

Ahmed, IH; Krachmarova, E; Nacheva, G; Ivanov, I. Nucleic acids in inclusion bodies obtained from E. coli cells expressing human interferon-gamma. In: Abstract book of the 8th Poster Session of the University of Chemical Technology and Metallurgy, Sofia, Bulgaria, 18 May 2011, p. 86.

Ahmed, MU; Brinkmann Frye, E; Degenhardt, TP; Thorpe, SR; Baynes, JW. N-epsilon-(carboxyethyl)lysine, a product of the chemical modification of proteins by methylglyoxal, increases with age in human lens proteins. *Biochem J*, 1997 Jun 1;324 (Pt 2):565-570.

Ahmed, MU; Thorpe, SR; Baynes, JW. Identification of N epsilon-carboxymethyllysine as a degradation product of fructoselysine in glycated protein. *J Biol Chem*, 1986 Apr 15;261(11):4889-4894.

Ahmed, N; Thornalley, PJ. Quantitative screening of protein biomarkers of early glycation, advanced glycation, oxidation and nitrosation in cellular and extracellular proteins by tandem mass spectrometry multiple reaction monitoring. *Biochem Soc Trans*, 2003 Dec;31(Pt 6):1417-1422.

Ajith, TA; Vinodkumar, P. Advanced Glycation End Products: Association with the Pathogenesis of Diseases and the Current Therapeutic Advances. *Curr Clin Pharmacol*, 2016;11(2):118-127.

Alexander, P. The role of DNA lesions in the processes leading to aging in mice. *Symp Soc Exp Biol*, 1967;21:29-50.

Arunasri, K; Adil, M; Khan, PA; Shivaji, S. Global gene expression analysis of long-term stationary phase effects in E. coli K12 MG1655. *PLoS One*, 2014 May 23;9(5):e96701.

Barker, M. G. and Walmsley R. M. Replicative ageing in the fission yeast Schizosaccharomyces pombe. *Yeast*, 1999 Oct;15(14):1511-1518.

Bartke, A.; Chandrashekar V. Reproductive Function in Long Lived Mice: Effects of Mutations and Caloric Restriction. *Annu Rev Biomed Sci*, 2003 Jan;5:69-78.

Barton, A. Some aspects of cell division in Saccharomyces cerevisiae. *J Gen Microbiol*, 1950 Jan;4(1):84-86.

Baynes, JW. From life to death-the struggle between chemistry and biology during aging: the Maillard reaction as an amplifier of genomic damage. *Biogerontology*, 2000;1(3):235-246.

Baynes, JW. The Maillard hypothesis on aging: time to focus on DNA. *Ann N Y Acad Sci*, 2002 Apr;959:360-367.

Bjorksten, J. A common molecular basis for the aging syndrome. *J Am Geriatr Soc*, 1958 Oct;8(10):740-748.

Bjorksten, J; Tenhu H. The crosslinking theory of aging-added evidence. *Exp Gerontol*, 1990;25(2):91-95.

Boyanova, M; Mironova, R; Niwa, T; Ivanov, I. Post-translational processing of human interferon-gamma and approaches for its prevention. In: Georgiev VS, Western KA, McGovan JJ editors. *Infectious disease*. Totowa, NJ, USA: Humana Press; 2008; 365- 373.

Brownlee, M. Advanced protein glycosylation in diabetes and aging. *Annu Rev Med*, 1995;46:223-234.

Bucala, R; Model, P; Cerami, A. Modification of DNA by reducing sugars: a possible mechanism for nucleic acid aging and age-related dysfunction in gene expression. *Proc Natl Acad Sci USA*, 1984 Jan;81(1):105-109.

Castellani, R; Smith, MA; Richey, PL; Perry, G. Glycoxidation and oxidative stress in Parkinson disease and diffuse Lewy body disease. *Brain Res*, 1996 Oct 21;737(1-2):195-200.

Cerami, A. Aging of proteins and nucleic acids: what is the role of glucose? *Trends Biochem Sci*, 1986, Aug;11(8):311-314.

Cohen-Or I, Katz C, Ron EZ. AGEs secreted by bacteria are involved in the inflammatory response. *PLoS One*, 2011 Mar 22;6(3):e17974.

Cohen-Or I, Katz C, Ron EZ. Metabolism of AGEs-bacterial AGEs are degraded by metallo-proteases. *PLoS One*, 2013 Oct 9;8(10):e74970.

Colman, RJ; Beasley, TM; Kemnitz, JW, Johnson, SC; Weindruch, R; Anderson RM. Caloric restriction reduces age-related and all-cause mortality in rhesus monkeys. *Nat Commun*, 2014 Apr 1;5:3557-3561.

Darwin, CR. On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life. 1st Edition. London: John Murray; 1859.

de Vos, LC; Lefrandt, JD; Dullaart, RP; Zeebregts, CJ; Smit AJ. Advanced glycation end products: An emerging biomarker for adverse outcome in patients with peripheral artery disease. *Atherosclerosis*, 2016 Nov;254: 291-299.

Delpierre, G; Collard, F; Fortpied, J; Van Schaftingen, E. Fructosamine 3-kinase is involved in an intracellular deglycation pathway in human erythrocytes. *Biochem J*, 2002 Aug 1;365(Pt 3):801-808.

Delpierre, G; Rider, MH; Collard, F; Stroobant, V; Vanstapel, F; Santos, H; Van Schaftingen E. Identification, cloning, and heterologous expression of

a mammalian fructosamine-3-kinase. *Diabetes*, 2000 Oct;49(10):1627-1634.

den Blaauwen, T; de Pedro, MA; Nguyen-Disteche, M; Ayala, JA. Morphogenesis of rod-shaped sacculi. *FEMS Microbiol Rev*, 2008 Mar;32 (2):321-344.

Dimitrova, R; Mironova, R; Ivanov, I. Glycation of proteins in Escherichia coli: Effect of nutrient broth ingredients on glycation. *Biotechnol Biotechnol Eq*, 2004;18(2):99-103.

Ericsson, M; Hanstorp, D; Hagberg, P; Enger, J; Nyström, T. Sorting out bacterial viability with optical tweezers. *J Bacteriol*, 2000 Oct;182(19): 5551-5555.

Ferguson, GP. Protective mechanisms against toxic electrophiles in Escherichia coli. *Trends Microbiol*, 1999 Jun;7(6):242-247.

Finkel, SE. Long-term survival during stationary phase: evolution and the GASP phenotype. *Nat Rev Microbiol*, 2006 Feb;4(2):113-120.

Fleming, T; Rabbani, N; Thornalley P. Quantitative screening of DNA biomarkers of glycation and oxidation. In: Abstract book of the 9th International Symposium on the Maillard Reaction, Munich, Germany, 1-5 September 2007, p. 99.

Fontaine, F; Stewart, EJ; Lindner, AB; Taddei, F. Mutations in two global regulators lower individual mortality in Escherichia coli. *Mol Microbiol*, 2008 Jan;67(1):2-14.

Freedberg, WB; Kistler, WS; Lin, ECC. Lethal synthesis of methylglyoxal by Escherichia coli during unregulated glycerol metabolism. *J Bacteriol*, 1971 Oct;108(1):137–144.

Friedman, DB; Johnson, TE. A mutation in the age-1 gene in Caenorhabditis elegans lengthens life and reduces hermaphrodite fertility. *Genetics*, 1988 Jan;118(1):75-86.

Gagliardi, A; Lamboglia, E; Bianchi, L; Landi, C; Armini, A; Ciolfi, S; Bini, L; Marri, L. Proteomics analysis of a long-term survival strain of Escherichia coli K-12 exhibiting a growth advantage in stationary-phase (GASP) phenotype. *Proteomics*, 2016 Mar;16(6):963-972.

Gallant, J; Palmer L. Error propagation in viable cells. *Mech Ageing Dev*, 1979 Apr;10(1-2):27-38.

Gemayel, R; Fortpied, J; Rzem, R; Vertommen, D; Veiga-da-Cunha, M; Van Schaftingen, E. Many fructosamine 3-kinase homologues in bacteria are ribulosamine/erythrulosamine 3-kinases potentially involved in protein deglycation. *FEBS J*, 2007 Sep;274(17):4360-74.

Handzhiyski, Y; Krasteva, M; Hristova, I; Ivanov, I; Mironova, R. Interaction of DNA with reducing sugars as a source of spontaneous mutations in the gut bacterium Escherichia coli. *J Bulg Acad Sci*, 2012;125(1):15-20. Article in Bulgarian.

Hanssen, NM; Wouters, K; Huijberts, MS; Gijbels, MJ; Sluimer, JC; Scheijen, JL; Heeneman, S; Biessen, EA; Daemen, MJ; Brownlee, M; de Kleijn, DP; Stehouwer, CD; Pasterkamp, G; Schalkwijk, CG. Higher levels of advanced glycation endproducts in human carotid atherosclerotic plaques are associated with a rupture-prone phenotype. *Eur Heart J*, 2014 May;35 (17):1137-1146.

Harman, D. Aging: a theory based on free radical and radiation chemistry. *J Gerontol*, 1956 Jul;11(3):298-300.

Hellwig, M; Henle T. Baking, ageing, diabetes: a short history of the Maillard reaction. *Angew Chem Int Ed Engl*, 2014 Sep 22;53(39):10316-10329.

Helou, C; Marier, D; Jacolot, P; Abdennebi-Najar, L; Niquet-Léridon, C; Tessier, FJ; Gadonna-Widehem, P. Microorganisms and Maillard reaction products: a review of the literature and recent findings. *Amino Acids*, 2014 Feb;46(2):267-277.

Henderson, ST; Johnson, TE. daf-16 integrates developmental and environmental inputs to mediate aging in the nematode Caenorhabditis elegans. *Curr Biol*, 2001 Dec 11;11(24):1975-1980. Erratum in: Curr Biol. 2005 Apr 12;15(7):690.

Higuchi-Sanabria, R; Pernice, WM; Vevea, JD; Alessi Wolken, DM; Boldogh, IR; Pon, LA. Role of asymmetric cell division in lifespan control in Saccharomyces cerevisiae. *FEMS Yeast Res*, 2014 Dec;14(8):1133-1146.

Hill, SM; Hao, X; Grönvall, J; Spikings-Nordby, S; Widlund, PO; Amen, T; Jörhov, A; Josefson, R; Kaganovich, D; Liu, B; Nyström, T. Asymmetric Inheritance of Aggregated Proteins and Age Reset in Yeast Are Regulated by Vac17-Dependent Vacuolar Functions. *Cell Rep*, 2016 Jul 19;16(3): 826-838.

Hodge, JE. The Amadori rearrangement. Adv Carbohydr Chem, 1955;10:169-205.

Hopper, DJ; Cooper, RA. The purification and properties of Escherichia coli methylglyoxal synthase. *Biochem J*, 1972 Jun;128(2):321-329.

Jana, AK; Batkulwar, KB; Kulkarni, MJ; Sengupta, N. Glycation induces conformational changes in the amyloid-β peptide and enhances its aggregation propensity: molecular insights. *Phys Chem Chem Phys*, 2016 Nov 23;18(46):31446-31458.

Kadner, RJ; Murphy, GP; Stephens, CM. Two mechanisms for growth inhibition by elevated transport of sugar phosphates in Escherichia coli. *J Gen Microbiol*, 1992 Oct;138(10):2007–2014.

Karanjawala, ZE; Lieber MR. DNA damage and aging. *Mech Ageing Dev*, 2004 Jun;125(6):405-416.

Katz, C; Cohen-Or, I; Gophna, U; Ron, EZ. The ubiquitous conserved glycopeptidase Gcp prevents accumulation of toxic glycated proteins. *MBio*, 2010 Aug 24;1(3). pii: e00195-10.

Kirkwood, TB. Evolution of ageing. *Nature*, 1977 Nov 24;270(5635):301-304.

Kirkwood, TB. Understanding the odd science of aging. *Cell*, 2005 Feb 25;120(4):437-447.

Ko, J; Kim, I; Yoo, S; Min, B; Kim, K; Park, C. Conversion of methylglyoxal to acetol by Escherichia coli aldoketo reductases. *J Bacteriol*, 2005 Aug;187(16):5782-5789.

Kosmachevskaya, OV; Shumaev, KB; Topunov, AF. Carbonyl Stress in Bacteria: Causes and Consequences. *Biochemistry (Mosc)*, 2015 Dec;80 (13):1655-1671.

Krachmarova, E; Nacheva, G; Ivanov I. Inclusion bodies obtained from E. coli cells expressing human interferon-gamma contain nucleic acids. In: Abstract book of the Anniversary Molecular Biology Conference "50 Year Roumen Tsanev Institute of Molecular Biology," Sofia, Bulgaria, 6-7 October 2011, p. 90.

Kram, KE; Finkel, SE. Culture volume and vessel affect long-term survival, mutation frequency, and oxidative stress of Escherichia coli. *Appl Environ Microbiol*, 2014 Mar;80(5):1732-1738.

Kram, KE; Finkel, SE. Rich Medium Composition Affects Escherichia coli Survival, Glycation, and Mutation Frequency during Long-Term Batch Culture. *Appl Environ Microbiol*, 2015 Jul;81(13):4442-4450.

Lapolla, A; Molin, L; Traldi, P. Protein glycation in diabetes as determined by mass spectrometry. *Int J Endocrinol*, 2013 Mar 13;2013:412103.

Lee, C; Lee, J; Lee, JY; Park, C. Characterization of the Escherichia coli YajL, YhbO and ElbB glyoxalases. *FEMS Microbiol Lett*, 2016 Feb;363(3). pii: fnv239.

Lee, SC; Chan, JC. Evidence for DNA damage as a biological link between diabetes and cancer. *Chin Med J (Engl)*, 2015 Jun 5;128(11):1543-1548.

Leroi, AM; Chippindale, AK; Rose, MR. Long-term laboratory evolution of a genetic life-history tradeoff in Drosophila melanogaster. The role of genotype-by-environment interaction. *Evolution*, 1994 Aug;48(4), 1244–1257.

Li, XH; Du, LL; Cheng, XS; Jiang, X; Zhang, Y; Lv, BL; Liu, R; Wang, JZ; Zhou, XW. Glycation exacerbates the neuronal toxicity of β-amyloid. *Cell Death Dis*, 2013 Jun 13;4:e673.

Lindner, A. B; Madden, R; Demarez, A; Stewart, EJ; Taddei, F. Asymmetric segregation of protein aggregates is associated with cellular aging and rejuvenation. *Proc Natl Acad Sci USA*, 2008 Feb 26;105(8):3076 –3081.

Linetsky, M; Shipova, E; Cheng, R; Ortwerth, BJ. Glycation by ascorbic acid oxidation products leads to the aggregation of lens proteins. *Biochim Biophys Acta*, 2008 Jan;1782(1):22-34.

MacLean, MJ; Ness, LS; Ferguson, GP; Booth, IR. The role of glyoxalase I in the detoxification of methylglyoxal and in the activation of the KefB K+ efflux system in Escherichia coli. *Mol Microbiol*, 1998 Feb;27(3):563-571.

Maillard, LC. Action des acides amines sur les sucres: formation des melanoidines par voie methodique. *C R Acad Sci*, 1912;154:66-68.

Medawar, PB. An Unsolved Problem of Biology. 1st Edition. London: Lewis; 1952.

Miller, MB; Bassler, BL. Quorum sensing in bacteria. *Annu Rev Microbiol*, 2001;55:165-199.

Mironova, R; Handzhiyski, Y; Niwa, T; Berzal-Herranz, A; Datsenko, KA; Wanner, BL; Ivanov, I. Maillard Reaction and Spontaneous Mutagenesis in Escherichia coli. In: Knutsen DW, Bruns SS editors. *Bacterial DNA, DNA Polymerase and DNA Helicases*. New York, USA: Nova Science Publishers, Inc.; 2009;51-89.

Mironova, R; Niwa, T; Dimitrova, R; Boyanova, M; Ivanov, I. Glycation and post-translational processing of human interferon-gamma expressed in Escherichia coli. *J Biol Chem*, 2003 Dec 19;278(51):51068-51074.

Mironova, R; Niwa, T; Handzhiyski, Y; Sredovska, A; Ivanov, I. Evidence for non-enzymatic glycosylation of Escherichia coli chromosomal DNA. *Mol Microbiol*, 2005 Mar;55(6):1801-1811.

Mironova, R; Niwa, T; Hayashi, H; Dimitrova, R; Ivanov I. Evidence for non-enzymatic glycosylation in Escherichia coli. *Mol Microbiol*, 2001 Feb;39 (4):1061-1068.

Misra, K; Banerjee, AB; Ray, S; Ray, M. Glyoxalase III from Escherichia coli: a single novel enzyme for the conversion of methylglyoxal into D-lactate without reduced glutathione. *Biochem J*, 1995 Feb 1;305(Pt 3):999-1003.

Misra, K; Banerjee, AB; Ray, S; Ray, M. Reduction of methylglyoxal in Escherichia coli K12 by an aldehyde reductase and alcohol dehydrogenase. *Mol Cell Biochem*, 1996 Mar 23;156(2):117-124.

Mogk, A; Deuerling, E; Vorderwülbecke, S; Vierling, E; Bukau, B. Small heat shock proteins, ClpB and the DnaK system form a functional triade in reversing protein aggregation. *Mol Microbiol*, 2003 Oct;50(2):585-595.

Monnier, VM. Toward a Maillard reaction theory of aging. *Prog Clin Biol Res*, 1989;304:1-22.

Mortimer, R. K. and Johnston, J. R. Life span of individual yeast cells. *Nature*, 1959 Jun 20;183(4677):1751-1752.

Münch, G; Lüth, HJ; Wong, A; Arendt, T; Hirsch, E; Ravid, R; Riederer, P. Crosslinking of alpha-synuclein by advanced glycation endproducts-an early pathophysiological step in Lewy body formation? *J Chem Neuroanat*, 2000 Dec;20(3-4):253-257.

Nagaraj, RH; Linetsky, M; Stitt, AW. The pathogenic role of Maillard reaction in the aging eye. *Amino Acids*, 2012 Apr;42(4):1205-1220.

Nakayama, T; Hayase, F; Kato, H. Formation of Nε-(2-formyl-5-hydroxy-methyl-pyrrol-1-yl)-L-norleucine in the Maillard reaction between D-glucose and L-lysine. *Agric Biol Chem*, 1980, Dec;44(5):1201-1202.

Nass, N; Bartling, B; Navarrete, Santos, A; Scheubel, RJ; Börgermann, J; Silber, RE; Simm, A. Advanced glycation end products, diabetes and ageing. *Z Gerontol Geriatr*, 2007 Oct;40(5):349-356.

Nyström, T. Aging in bacteria. *Curr Opin Microbiol*, 2002 Dec;5(6):596-601.

Nyström, T. Conditional senescence in bacteria: death of the immortals. *Mol Microbiol*, 2003 Apr;48(1):17-23.

Nyström, T. Starvation, cessation of growth and bacterial aging. *Curr Opin Microbiol*, 1999 Apr;2(2):214-219.

Nyström, T; Liu, B. The mystery of aging and rejuvenation - a budding topic. *Curr Opin Microbiol*, 2014 Apr;18:61-67.

Pageon, H. Reaction of glycation and human skin: the effects on the skin and its components, reconstructed skin as a model. *Pathol Biol (Paris)*, 2010 Jun;58(3):226-231.

Pepper, ED. (2007). Mechanisms of Long-Term Survival in Escherichia coli: PhD thesis (Molecular Biology), University of Southern California, USA, p. 87.

Pepper, ED; Farrell, MJ; Nord, G; Finkel, SE. Antiglycation effects of carnosine and other compounds on the long-term survival of Escherichia coli. *Appl Environ Microbiol*, 2010 Dec;76(24):7925-7930.

Pescosolido, N; Barbato, A; Giannotti, R; Komaiha, C; Lenarduzzi, F. Age-related changes in the kinetics of human lenses: prevention of the cataract. *Int J Ophthalmol*, 2016 Oct 18;9(10):1506-1517.

Pongor, S; Ulrich, PC; Bencsath, FA; Cerami, A. Aging of proteins: isolation and identification of a fluorescent chromophore from the reaction of polypeptides with glucose. *Proc Natl Acad Sci USA*, 1984 May;81(9): 2684-2688.

Popov, M; Petrov, S; Nacheva, G; Ivanov, I; Reichl, U. Effects of a recombinant gene expression on ColE1-like plasmid segregation in Escherichia coli. *BMC Biotechnol*, 2011 Mar 1;11:18.

Popov, MN. (2012). Effects of Human Interferon-gamma Gene Expression and Structure of ColE1-like Plasmids on Plasmid Segregation in Escherichia coli: PhD thesis (Molecular Biology), Institute of Molecular Biology "Roumen Tsanev," Bulgarian Academy of Sciences, Bulgaria, p. 66.

Rabbani, N; Xue, M; Thornalley, PJ. Methylglyoxal-induced dicarbonyl stress in aging and disease: first steps towards glyoxalase 1-based treatments. *Clin Sci (Lond)*, 2016 Oct 1;130(19):1677-1696.

Rahbar S. The discovery of glycated hemoglobin: a major event in the study of nonenzymatic chemistry in biological systems. *Ann N Y Acad Sci*, 2005 Jun;1043:9-19.

Rahbar, S. An abnormal hemoglobin in red cells of diabetics. *Clin Chim Acta*, 1968 Oct;22(2):296-298.

Rang, CU; Peng, AY; Chao, L. Temporal dynamics of bacterial aging and rejuvenation. *Curr Biol*, 2011 Nov 8;21(21):1813-1816.

Rekarte, UD; Zwaig, N; Istriz, T. Accumulation of methylglyoxal in a mutant of Escherichia coli constitutive for gluconate catabolism. *J Bacteriol*, 1973 Sep;115(3):727–731.

Rose, M. (1991) Evolutionary biology of aging. 1st Edition. New York; Oxford University Press.

Sadowska-Bartosz, I; Bartosz, G. Effect of glycation inhibitors on aging and age-related diseases. *Mech Ageing Dev*, 2016 Dec;160:1-18.

Saikusa, T; Rhee, H; Watanabe, K; Murata, K; Kimura, A. Metabolism of 2- oxoaldehydes in bacteria: purification and characterization of methylglyoxal reductase from Escherichia coli. *Agric Biol Chem*, 1987 Jan;51(7):1893-1899.

Saint-Ruf, C; Garfa-Traoré, M; Collin, V; Cordier, C; Franceschi, C; Matic, I. Massive diversification in aging colonies of Escherichia coli. *J Bacteriol*, 2014 Sep;196(17):3059-3073.

Sell, DR; Monnier, VM. Structure elucidation of a senescence cross-link from human extracellular matrix. Implication of pentoses in the aging process. *J Biol Chem*, 1989 Dec 25;264(36):21597-21602.

Stewart, EJ; Madden, R; Paul, G; Taddei, F. Aging and death in an organism that reproduces by morphologically symmetric division. *PLoS Biol*, 2005 Feb;3(2):e45.

Subedi, KP; Choi, D; Kim, I; Min, B; Park, C. Hsp31 of Escherichia coli K-12 is glyoxalase III. *Mol Microbiol*, 2011 Aug;81(4):926-936.

Thornalley, PJ; Battah, S; Ahmed, N; Karachalias, N; Agalou, S; Babaei-Jadidi, R; Dawnay, A. Quantitative screening of advanced glycation endproducts in cellular and extracellular proteins by tandem mass spectrometry. *Biochem J*, 2003 Nov 1;375(Pt 3):581-592.

Thorpe, SR; Baynes, JW. Maillard reaction products in tissue proteins: new products and new perspectives. *Amino Acids*, 2003 Dec;25(3-4):275-281.

Thorpe, SR; Baynes, JW. Role of the Maillard reaction in diabetes mellitus and diseases of aging. *Drugs Aging*, 1996 Aug;9(2):69-77.

Tötemeyer, S; Booth, NA; Nichols, WW; Dunbar, B; Booth IR. From famine to feast: the role of methylglyoxal production in Escherichia coli. *Mol Microbiol*, 1998 Feb;27(3):553-562.

Tsanev, R. and Ivanov, I. (2002). Immune interferon (1st edition). New York, USA: CRC Press.

Ulrich, P; Cerami, A. Protein glycation, diabetes, and aging. *Recent Prog Horm Res*, 2001;56:1-21.

Verzijl, N; DeGroot, J; Thorpe, SR; Bank, RA; Shaw, JN; Lyons, TJ; Bijlsma, JW; Lafeber, FP; Baynes, JW; TeKoppele, JM. Effect of collagen turnover on the accumulation of advanced glycation end products. *J Biol Chem*, 2000 Dec 15;275(50):39027-39031.

Vicente Miranda, H; El-Agnaf, OM; Outeiro, TF. Glycation in Parkinson's disease and Alzheimer's disease. *Mov Disord*, 2016 Jun;31(6):782-790.

Vulic, M; Kolter, R. Evolutionary cheating in Escherichia coli stationary phase cultures. *Genetics*, 2001 Jun;158(2):519-26.

Wang, P; Robert, L; Pelletier, J; Dang, WL; Taddei, F; Wright, A; Jun, S. Robust growth of Escherichia coli. *Curr Biol*, 2010 Jun 22;20(12):1099-1103.

Wiame, E; Delpierre, G; Collard, F; Van Schaftingen, E. Identification of a pathway for the utilization of the Amadori product fructoselysine in Escherichia coli. *J Biol Chem*, 2002 Nov 8;277(45):42523-42529.

Williams, GC. (1957). Pleiotropy, natural selection and the evolution of senescence. *Evolution*, 1957 11:398–411.

Winkler, J; Seybert, A; Konig, L; Pruggnaller, S; Haselmann, U; Sourjik, V; Frangakis, AS; Mogk, A; Bukaru, B. (2010). Quantitative and spatio-temporal features of protein aggregation in Escherichia coli and

consequences on protein quality control and cellular ageing. *EMBO J*, 2010 Mar 3;29(5):910-923.

Yuan, B; Cao, H; Jiang, Y; Hong, H; Wang, Y. Efficient and accurate bypass of N2-(1-carboxyethyl)-2'-deoxyguanosine by DinB DNA polymerase in vitro and in vivo. *Proc Natl Acad Sci USA*, 2008 Jun 24;105(25):8679-8684.

Zambrano, MM; Kolter, R. GASPing for life in stationary phase. *Cell*, 1996 Jul 26;86(2):181-184.

Zambrano, MM; Siegele, DA; Almirón, M; Tormo, A; Kolter, R. Microbial competition: Escherichia coli mutants that take over stationary phase cultures. *Science*, 1993 Mar 19;259(5102):1757-1760.

Zinser, ER; Kolter R. Mutations enhancing amino acid catabolism confer a growth advantage in stationary phase. *J Bacteriol*, 1999 Sep;181(18): 5800-5807.

Zinser, ER; Kolter, R. Escherichia coli evolution during stationary phase. *Res Microbiol*, 2004 Jun;155(5):328-336.

Zinser, ER; Kolter, R. Prolonged stationary-phase incubation selects for lrp mutations in Escherichia coli K-12. *J Bacteriol*, 2000 Aug;182(15):4361-4365.

Zinser, ER; Schneider, D; Blot, M; Kolter, R. Bacterial evolution through the selective loss of beneficial Genes. Trade-offs in expression involving two loci. *Genetics*, 2003 Aug;164(4):1271-1277.

Reviewed by
Lachezar A. Nikolov
Department of Comparative Development and Genetics,
Max Planck Institute for Plant Breeding Research, Köln, Germany

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Chapter 6

HAPLOINSUFFICIENT TUMOR SUPPRESSOR GENES

Kazushi Inoue [*] ***and Elizabeth A. Fry***
The Department of Pathology, Wake Forest University Health Sciences,
Medical Center Boulevard, Winston-Salem, NC 27157 US

ABSTRACT

Haploinsufficiency of tumor suppressor genes (TSGs) indicates that the reduced levels of proteins in cells that lack one allele of the genomic locus results in the inability of the cell to execute normal cellular functions contributing to tumor development. Representative cases of haploinsufficient TSGs are $p27^{Kip1}$, *p53*, *DMP1*, *NF1*, and *PTEN*. Tumor development is significantly accelerated in both mice with homozygous and heterozygous gene deletion, with expression of the wild type allele in the latter. Newly characterized TSGs such as *AML1*, *EGR1*, *TGFβR1/2*, and *SMAD4* have also shown haploid insufficiency for tumor suppression. This phenotype has typically been demonstrated in gene knockout mouse models, but analyses of human samples have been conducted in some cases. Recent studies suggest collaboration of multiple haploinsufficient TSGs in 5q-, 7q-, and 8q- syndromes, which is called compound haploinsufficiency. Although *ARF* is a classical TSG, it also belongs to this category since $Arf^{+/-}$ accelerates tumor development when both alleles for *Ink4a* are inactivated. Haploid insufficiency of *Arf* was

[*] Corresponding Author E-mail: kinoue2@triad.rr.com, EAF e-mail: eafry10@hotmail.com.

also reported in myeloid leukemogenesis in the presence of inv(16). In case of p53, *p53*$^{+/-}$ cells achieve only ~25% of p53 mRNA and protein levels as compared to those in wild type, which could explain the mechanism. *TGFβR1*$^{+/-}$ collaborates with *Apc*$^{Min+/-}$ in colorectal cancer development; *TGFβR2*$^{+/-}$ and *Smad4*$^{+/-}$ collaborates with *K-Ras* mutation in pancreatic ductal adenocarcinomagenesis, demonstrating the synergism of haploinsufficient TSGs and other oncogenic events. These TSGs can be targets for activation therapy in cancer since they retain a functional allele even in tumor cells.

Keywords: haploinsufficiency, tumor suppressor gene, p27^{Kip1}, p53, DMP1 (DMTF1), ARF, AML1, EGR1, TGFβ/TGFβR/SMAD4, mouse model

INTRODUCTION

Cancer is a complex genetic disorder caused by alterations for both gene coding and non-coding regions. Two major classes of genes have been identified, namely oncogenes and tumor suppressor genes (TSGs). For instance, overexpression of a proto-oncogene, such as c-*Myc*, and/or inactivation of a tumor suppressor, such as *TP53* or *RB* induces tumors. Oncogene activation in tumors is relatively straightforward since they are overexpressed or activated by mutation(s) while inactivation of a TSG is a complicated process performed by different mechanisms, e.g., gene deletion, mutation, epigenetic silencing, abnormal activity of microRNAs (miRNAs), and/or aberrant splicing. Although complete loss of TSG is common in human cancers as predicted by the Knudson's two-hit hypothesis [1], recent studies indicate that an incomplete process for TSG function termed ‘haploinsufficiency’ that contributes to the development and progression of many cancers (Figure 1). In this category of TSG, one functional allele of a gene is lost by mutation or deletion while the remaining normal allele is still retained in tumors, but the activity is not enough to execute its physiological function to prevent abnormal cell proliferation. Promoter hypermethylation or point mutation does not happen in the retained locus in haploinsufficient TSGs, thus tumor cells still express the wild type mRNA. We use this definition of haploinsufficient TSG throughout this review.

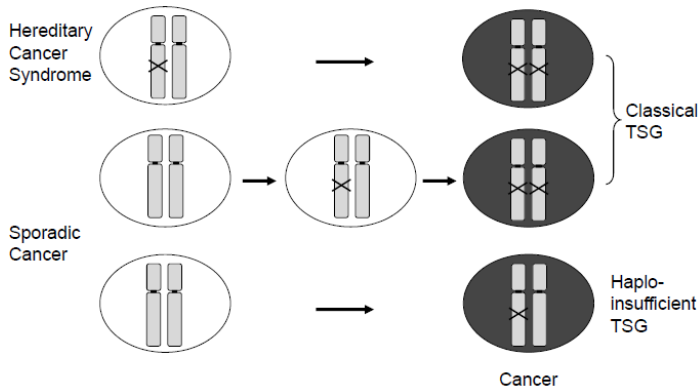

Figure 1. Tumor development caused by classical and haploinsufficient tumor suppressor genes.

Top panel: In case of hereditary cancer syndrome (e.g., Li-Fraumeni syndrome), one TSG locus is mutated in all the chromosomes, and inactivation of the intact allele leads to cancer following the two-hit hypothesis by Dr. Knudson [1]. Tumor development is much accelerated because only a single hit on the TSG locus leads to total inactivation of the gene leading to carcinogenesis.

Middle panel: Tumor development in classical TSGs. It will take longer than the above for the tumor to develop since biallelic inactivation of the locus is needed.

Lower panel: Tumor development in haploinsufficient TSGs. It will take more time for tumor(s) to develop than classical TSGs since collaboration of mono-allelic loss with other genetic alteration(s) are required.

The Cdk inhibitor *p27*^{kip1} [2] was the first gene to be characterized as haploinsufficient for tumor suppression, followed by *Dmp1* [3, 4, reviewed in 5] through observation of tumor development in homozygous and heterozygous knockout mice. *Pten* and *Nf1* are also haploinsufficient TSGs since mice with heterozygous genomic DNA deletion often develop tumors without deletion of the wild type allele; however, comparative tumor development assays were not possible using global knockout mice due to the lethality of homozygous gene deletion [6-8, reviewed in 9]. Creation of prostate-specific gene deletion in mice showed that complete loss of *Pten* expression activates a p53-dependent cellular senescence that can act as a brake on tumor formation [10]. Hence the genomic locus for *PTEN* needs to be mono-allelic loss to promote prostate carcinogenesis, raising the concept of 'obligate haploinsufficiency' [9]. TGFβ was shown to be haploinsufficient for tumor suppression [11]; however the situation is different from others since it

is a secreted protein. Although p53 has been classified as a classical TSG, haploid insufficiency of *p53* has also been demonstrated in mice [12] as well as in humans [13] from analyses of tumor samples of Li-Fraumeni syndrome patients.

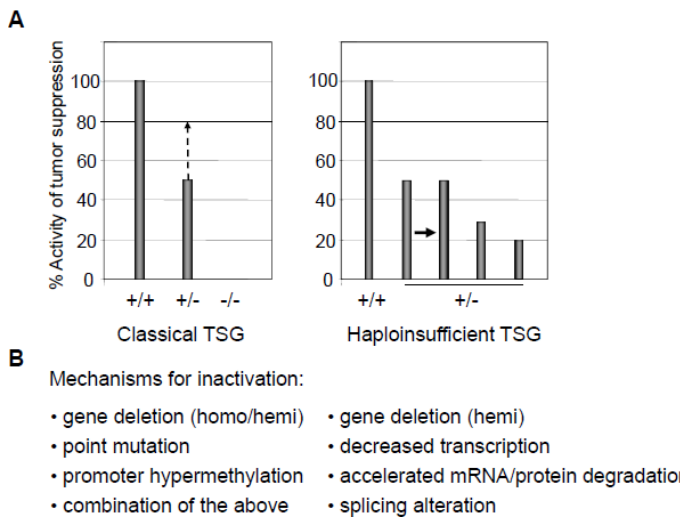

Figure 2. The activity of tumor suppressor genes/proteins in gene knockout mice (A) and possible mechanisms for inactivation of TSGs (B).

A. The wild type activity represents 100% diploid gene function and null (-/-) represents complete loss of function of the TSG. In classical TSGs, both loci are inactivated by i) point mutation, ii) gene deletion, iii) promoter hypermethylation, or iv) any combinations of these during tumor development. In this case, loss of one locus leads to compensatory activation of the remaining allele, leading to more than 50% of the tumor suppressor activity (left columns, middle, discontinued line; ref. 124). In haploinsufficient TSGs, tumors from mice with heterozygous gene deletion express the mRNA/protein at levels ~50% of wild type cells. The levels may be lower if other inactivation mechanisms co-exist to lower the levels of the tumor suppressive mRNA/protein (right columns). In $p53^{+/-}$ cells, both mRNA and protein levels are reduced to ~25% of those in wild type cells [48].

B. Possible mechanisms for inactivation of classical and haploinsufficient TSGs. Classical TSGs are inactivated by homozygous or heterozygous gene deletion plus point mutation or promoter hypermethylation(s). Conversely, haploinsufficient TSGs are inactivated by hemizygous gene deletion or point mutation at the single locus without promoter methylation or mutation of the remaining allele. Recent studies show that other mechanisms, such as decreased transcription, accelerated RNA degradation [20], enhanced protein degradation, or aberrant splicing [72, 74, 119, 125-127] can coexist to down-regulate the TSG mRNA/protein expression to less than the 50% level of that in wild type cells in subsets of malignancies.

In case of classical TSGs (e.g., *RB*, *p53*, and *INK4a/ARF*), both loci are inactivated by i) point mutation, ii) gene deletion, iii) promoter hypermethylation, or iv) any combinations of these during tumor development (Figure 2A). Knockout mouse models for such TSGs usually exhibit obvious tumor-prone phenotype without carcinogenic challenge than those for haploinsufficient TSGs [14-16, 17, 18 for reviews]. Conversely, a study from Pandolfi's lab showed that even a 20% reduction of *PTEN* protein level contributed to the development of cancer [19]. When targeted by shRNAs, *p53* knockdown mice showed distinct phenotypes ranging from hyperplasia to malignancy depending on the expression levels in its protein [20]. Based on such observations, Berger et al. proposed a continuum model that accounts for subtle dosage effects of tumor suppressors including their regulation by miRNAs [21]. The dosage and function of haploinsufficient TSGs are critical, and understanding the impact of haploinsufficiency is important for assessment of inter-individual genetic variation as well as the molecular mechanism of haploinsufficient disorders.

Haploinsufficiency of multiple genes cooperate to promote tumorigenesis, a phenomenon called 'compound haploinsufficiency'. The 5q deletion (5q-) and 7q deletion (7q-) syndromes are typical examples of compound haploinsufficiency [22, 23], which demonstrate the importance of combinatorial interactions to elicit specific phenotypes. Deletion of chromosome 8p is also very common in human cancer. Using a murine model of hepatocellular carcinoma and *in vivo* RNAi, Xue et al. silenced the genes frequently deleted on human 8p22 and showed that multiple genes on chromosome 8p (*Dlc1*, *Vps37a*, *Fgl1*) could cooperatively inhibit tumorigenesis in mice predicting poor survival [24].

Although haploinsufficient TSGs do not have promoter hypermethylation or mutation(s) in the coding region for the retained locus, the mRNA and/or protein expression levels may decrease to less than 50% (usually 20-30%) of those in wild type cells (Figure 2A). We include these TSGs into the category of haplo-insufficient TSGs since the protein levels in $TSG^{+/-}$ mice never reach 0%. The possible mechanisms for partial inactivation of the wild type locus include 1) decreased transactivation of the promoter due to autoregulation, 2) accelerated degradation of mRNAs or proteins, and 3) splicing alterations (Figure 2B). For 2), recent research indicated that single nucleotide polymorphisms (SNPs) in the 3' untranslated region of mRNAs and miRNAs seed sequences that may cause haploinsufficiency at the level of mRNAs through altered binding specificity of miRNAs [20]. Networking analysis suggested that the haploinsufficient TSGs strongly interacted with one

another, and any subtle alterations in this network could contribute to tumorigenesis. A typical case for 3) is a transcription factor Dmp1 [3, 4], which will be discussed in this review.

In this chapter, we focus on the identification and characterization of early generation haploinsufficient TSGs, such as *p27^{Kip1}*, *p53*, *DMP1*, *TGFβ*; and then on recently characterized TSGs, such as *53BP1*, *AML1*, *EGR1*, and *TGFβR/SMAD4*. Although *ARF* is a classical TSG, it behaves like a haploinsufficient TSG under specific circumstances. Finally, we discuss future directions and therapeutic values for the research on haploinsufficient TSGs.

p27Kip1

Entry, progression, and exit from the G1 phase of the mammalian cell cycle in response to mitogens are governed by cyclin-dependent kinases (Cdks) regulated by the D- and E-type cyclins [25, 26]. p27^{Kip1} was first discovered as a key regulator of cell proliferation [canonical function; ref. 27, 28]. In addition to its initial identification as a CDK inhibitor, p27^{Kip1} has also emerged as a multifunctional protein with numerous non-canonical, CDK-independent functions that exert influence on key processes such as cell cycle regulation, cytoskeletal dynamics and cellular plasticity, cell migration, and stem-cell proliferation and differentiation [29]. While both p21^{Cip1} and p27^{Kip1} bind directly to cyclin/Cdk complexes to inhibit their Cdk activity and block cell proliferation, they are essential activators of cyclin D-dependent kinases in murine fibroblasts, thus are called 'assembly factors' [‘AF’ in Figure 3; ref. 30]. Abnormally low levels of the p27^{Kip1} protein are frequently found in human carcinomas, and these low levels correlate directly with both histological aggressiveness and patient mortality [2]. The *p27^{KIP1}* gene was mapped to chromosome band 12p13 that is hemizygously deleted in leukemias without mutations, which may confer a growth advantage to leukemic cells [31]. Fero et al. showed that that both *p27$^{Kip1+/-}$* and *p27$^{Kip1-/-}$* mice were predisposed to tumors in multiple tissues (intestinal and lung adenomas, pituitary tumors) when challenged with γ-irradiation or a potent DNA alkylating agent N-ethyl-nitrosourea [ENU, Table 1; ref. 2]. Therefore p27^{Kip1} is a multiple-tissue tumor suppressor in mice. Molecular analyses of tumors in *p27$^{Kip1+/-}$* mice show that the remaining wild type allele is neither mutated nor silenced. Hence, p27^{Kip1} is haploinsufficient for tumor suppression [2].

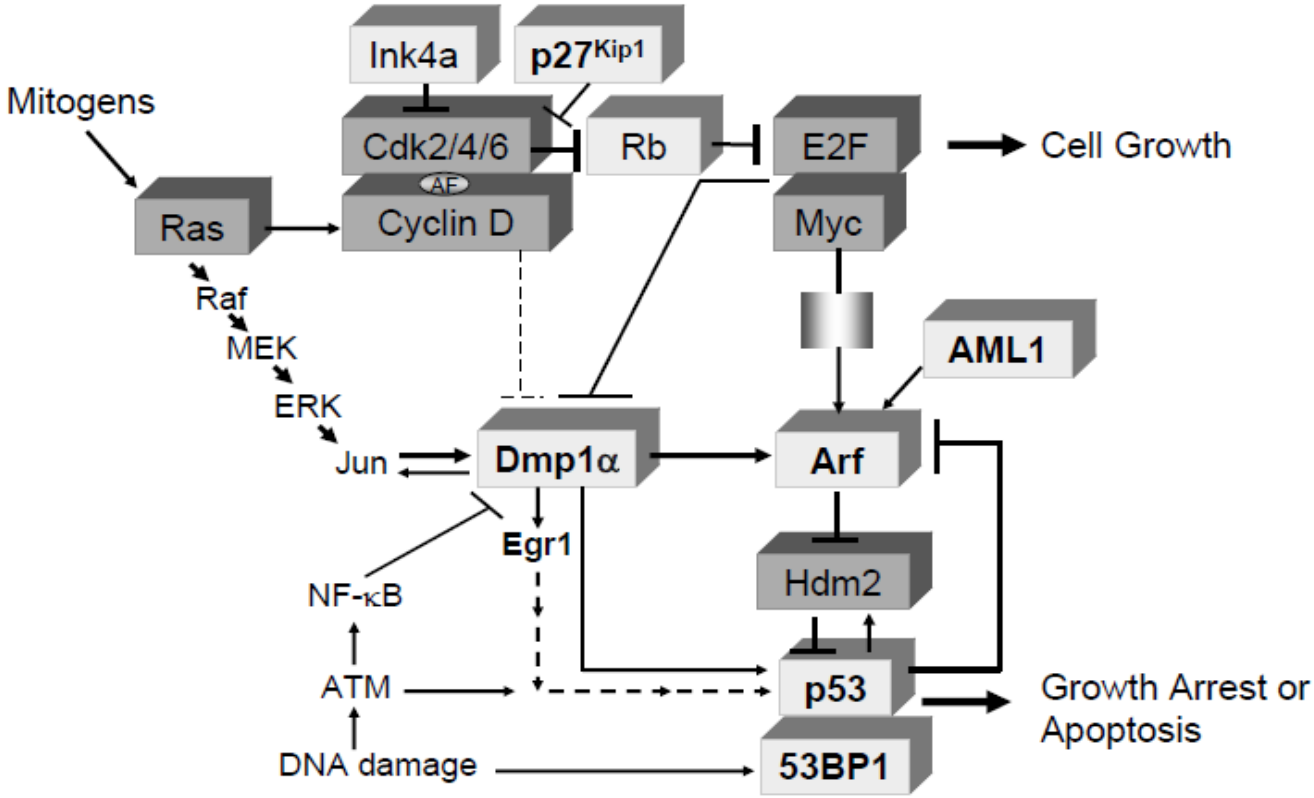

Figure 3. Intracellular signaling pathways involving p27Kip1, Dmp1, Arf, p53, 53BP1, AML1, and Egr1.

Cyclin D/Cdks are activated in response to mitogenic signals and initiate phosphorylation of Rb, a process that is completed by cyclin E - Cdk2. Once cells enter the S phase, cyclin E is degraded and cyclin A enters into complexes with Cdk2. Proteins of the Ink4 family (p15, p16, p18, p19) bind only to Cdk4 and inhibit its activity while those in the Kip/Cip family (p21, p27, p57) inhibit cyclin-bound Cdks [25-28]. Both $p21^{Cip1}$ and $p27^{Kip1}$ act as an assembly factor (AF; ref. 30). Arf is induced by potentially oncogenic signals stemming from overexpression of oncogenes such as c-Myc, E2F1, and activated Ras, which quenches inappropriate mitogenic signaling by diverting incipient cancer cells to undergo p53-dependent growth arrest or cell death [76, 77]. Dmp1 is activated by oncogenic Ras or HER2, which, in turn, binds and activates the *Arf* promoter and induces cell cycle arrest [61, 65]. Three different splicing variants have been reported for human *DMP1* [72, 73]. Dmp1 (Dmp1α) directly binds to the *CD13* [128] and *Arf* [56] promoters to transactivate the gene expression, thereby accelerates myeloid cell differentiation by interfering cell cycle progression. Interestingly the *CD13* promoter activation is inhibited by D-type cyclins in a Cdk-independent fashion [54, 128; see the dotted line for inhibition] while the *Arf* promoter activation is stimulated by cyclin D1 [129] in a Cdk-dependent fashion [57, 61]. Other transcriptional targets for Dmp1α include *Areg*, *Thbp*-1, *JunB*, and *Egr1* [118], suggesting that it is also involved in signal transduction related to angiogenesis and/or metastasis. Dmp1α physically interacts with p53 and neutralizes all the activities of Mdm2 to activate the p53 pathway [59, 60]. Both $Dmp1^{-/-}$ and $Dmp1^{+/-}$ mice show hypersensitivity to develop tumors in response to carcinogen or γ-irradiation. E2F1-3a [130, 131] directly binds to the *Dmp1* promoter and causes transcriptional repression [62]. The *Dmp1* promoter is repressed by NF-κB through direct binding of the promoter to RelA [63]. Conversely, the Dmp1 promoter is activated by the oncogenic Ras-Raf-MEK-ERK-Jun [61] and HER2-Pi3k-Akt-NF-κB [65] pathways, and thus Ras or HER2-driven carcinogenesis is dramatically accelerated in *Dmp1*-null mice. The transcription factor *AML1* locus is frequently translocated to create hybrid molecules in human acute leukemias, and plays essential roles in normal hematopoiesis through dimerization of its partner CBFβ [91, 92]. Direct transcriptional activation of the *ARF* promoter by AML1 and its inhibition by AML1-ETO has been reported [96] 53BP1 is a p53-binding protein, and is involved in DNA-damage response by choosing recombination and end joining at DNA double-strand breaks [50-52]. Haploid insufficiency of $p27^{Kip1}$, *p53, 53BP1, Dmp1α, Arf, AML1*, and *Egr1 TSGs* are discussed in this review. These genes are shown in bold. The *DMP1* locus generates other two splice variants, namely DMP1β and γ, and the oncogenic role of DMP1β has recently been demonstrated *in vivo* [72-74]. Aberrant splicing of TSGs and their roles in carcinogenesis are currently extensively studied [119, 125-127], which can further affect the function of the remaining allele of haploinsufficient TSGs. Proteins that have mitogenic/oncogenic functions are shown in dark while those with tumor-suppressive activities are shown in white.

ErbB2/neu destabilizes $p27^{Kip1}$ and increases the expression of cyclin D1. Muraoka et al. studied the roles of $p27^{Kip1}$ and cyclin D1 in ErbB2-mediated mammary epithelial cell transformation [32]. Overexpression of ErbB2 or cyclin D1 in $p27^{Kip1+/-}$ primary murine mammary epithelial cells resulted in increased proliferation, cyclin D1 nuclear localization, and colony formation in soft agar as compared to those in $p27^{Kip1+/+}$ cells. In contrast, ErbB2- or cyclin D1-overexpressing $p27^{Kip1-/-}$ cells displayed reduced proliferation, anchorage-independent growth, Cdk4 activity, and nuclear cyclin D1 expression compared to wild type cells. Mammary glands from *MMTV* (mouse mammary tumor virus)-*neu;* $p27^{Kip1+/-}$ mice exhibited alveolar hyperplasia, enhanced proliferation, decreased apoptosis, and accelerated tumor formation compared to *MMTV-neu;* $p27^{Kip1+/+}$ glands. Interestingly, *MMTV-neu;* $p27^{Kip1-/-}$ glands showed decreased proliferation, cyclin D1 expression, and Cdk4 activity, as well as markedly prolonged tumor latency, compared to *MMTV-neu;* $p27^{Kip1+/+}$ glands. Therefore $p27^{Kip1+/-}$ mammary epithelium are more susceptible to HER2/neu-induced tumorigenesis while $p27^{Kip1}$-null glands, due to severely impaired cyclin D1/Cdk4 function, are more resistant to transformation [32], suggesting another case of obligate haploinsufficiency.

Haploid insufficiency of $p27^{KIP1}$ was also demonstrated in T-cell prolymphocytic leukemia (T-PLL, 33) although hematopoietic malignancies were not reported in $p27^{Kip1-/-}$ mice. T-PLL is consistently associated with inactivation of the *ATM* gene and chromosomal rearrangements leading to an overexpression of the MTCP1/TCL1 oncoprotein that interact with AKT1. These alterations are present at the earliest stage of malignant transformation, suggesting that additional events are required for overt malignancy. Toriellec et al. studied the 12p13 deletion that occurs in approximately half of T-PLLs where found that the $p27^{KIP1}$ gene was hemizygously deleted in leukemic cells without mutation [33]. Consistently, in a $p27^{Kip1+/-}$ background, *MTCP1*-transgenics (TGs) had multiple emergences of preleukemic clones not observed in control cohorts. The remaining $p27^{Kip1}$ allele was maintained and expressed in these preleukemic clones, strongly implicating that $p27^{Kip1}$ was haploinsufficient in the pathogenesis of T-PLL [33]. Haploid insufficiency of $p27^{Kip1}$ for tumor suppression was also reported in human acute myeloid leukemia with complex karyotype [34], human small intestinal neuroendocrine tumors [35], mouse models for medulloblastoma [36] and pancreatic cancer [37], thus acceleration of tumorigenesis has been considered to be a generalized phenotype for heterozygous loss of $p27^{Kip1}$.

P53 AND 53BP1

p53 is an established tumor suppressor that is activated on cellular stresses such as DNA damage, oncogene activation, hypoxia, which transactivates sets of genes that induce cell cycle arrest, apoptosis, DNA repair, or autophagy, playing essential roles in the prevention of tumor development [38-40]. It is mutated or deleted in over ~50% of human cancers [41], but is inactivated in others with wild type p53 through different mechanisms, indicating that functional inactivation of the p53 pathway is essential for tumor development. Loss of heterozygosity (LOH) of *p53* often occurs in tumors having a *p53* mutation indicating that it behaves as a classical TSG that meets the Knudson's two-hit hypothesis [1]. However, *p53* is also a representative example of haploinsufficient TSG [12]. The first evidence that *p53* may exhibit haploinsufficiency comes from the analysis of Li-Fraumeni syndrome (LFS) patients [13, 42]. LFS is a hereditary cancer predisposition syndrome in whom a variety of cancers are found in affected families, having a higher risk of developing cancer, and the relatively early age of cancer development [42]. The majority of LFS cases are caused by germline missense mutations for *p53* [42, 43]. The most common types of cancer found in families with LFS include osteosarcoma, soft tissue sarcoma, acute leukemia, breast/lung/ gastrointestinal cancer, brain tumor, and adrenocortical tumor. When tumors from LFS patients were analyzed for LOH of *p53*, only half of the tumors exhibited LOH at the locus [13], raising the question of whether *p53* mutation acted as a dominant-negative inhibitor of the wild type allele [44] or if in fact single-copy loss of *p53* could contribute to tumorigenesis. In case of a dominant-negative mutation for *p53*, the wild type allele does not need to be lost to develop tumors since proteins from the mutant locus subvert the function of the wild type allele by making non-functional complexes because i) p53 proteins form tetramers to act as a transcription factor [45], ii) the level of Mdm2 is very low in p53-mutant cells, which results in accumulation of the protein to extremely high levels [46], and 3) degradation of mutant p53 is selectively compromised in tumor cells [46]. As a consequence, tetramers from $p53^{wt/mut}$ cells will almost exclusively be composed of mutant p53 that are non-functional. In case of a haploinsufficient mutation, the wild type *p53* allele is retained because half the normal wild type protein is insufficient to maintain the functionality. Since *p53* mutations from LFS patients often affect the oligomerization domain, the proteins remain unfolded and unable to interact with wild type p53; hence the locus is considered to be haploinsufficient than having a dominant-negative activity [47].

Table 1. The summary of haploinsufficient TSGs explained in this review. Genes for which evidence of haploinsufficiency comes from mouse and/or human studies are shown.

Genes	Human syndromes	Mouse Model	Cancer types	References
$p27^{Kip1}$		homo, het with γ-irradiation, ENU	intestinal adenoma, lung adenoma, pituitary tumor female reproductive tract tumor	[2]
		het;MMTV-neu TG, het;MTCP1 TG, het;Ptc+/-	mammary tumor, T-PLL, AML, small intestinal neuroendocrine tumor, medulloblastoma, pancreatic cancer	[32-37]
p53		homo	T-cell lymphoma, sarcoma, hemangiosarcoma	[14]
	LFS	het	soft tissue sarcoma, osteosarcoma, carcinoma	[12]
53BP1		homo, het	lymphoma, glioblastoma multiforme	[50, 53]
DMP1	7q-syndrome	homo, het with DMBA, gamma-irradiation	lung cancer, T cell lymphoma, ovarian tumor, liver tumor	[3, 4]
		Eμ-Myc, K-Ras^{LA}, MMTV-neu, cyclin D1	B cell lymphoma, lung cancer, mammary tumor	[4, 57, 64, 65]
ARF		homo	sarcoma, lymphoma, carcinoma, glioma	[16, 78]
		het	lymphoma, hemangioma, sarcoma, carcinoma	[136]
	FAMMM	het;Ink4a-/-	melanoma and other tumors	[79]
AML1	FPD/AML	het (homo lethal)	predisposition to myeloid leukemia	[93-98]
EGR1	5q-syndrome	het, homo with ENU	MPD, T cell lymphoma	[103]
		het;Apc+/-, het;p53+/-	t-MNs, AML	[106, 107]
TGFβ RI		het;$Apc^{Min/+}$ (homo lethal)	intestinal tumor	[110]
		het;$Kras^{G12D}$	PDAC	[113]
SMAD 4	Juvenile Polyposis	het (homo lethal)	gastrointestinal polyposis, intestinal tumor	[114, 115]
		het;$Kras^{G12D}$	MCNs developing into invasive PDAC	[116]

LFS: Li-Fraumeni syndrome; FAMMM: familial atypical multiple mole melanoma syndrome; FPD: familial platelet disorder; Homo: homozygous knockout; Het: heterozygous knockout; ENU: N-ethyl-nitrosourea; T-PLL: T-cell prolymphocytic leukemia; MTCP1: mature T cell proliferation-1; MPD: myeloproliferative disorder; t-MN: therapy-related myeloid neoplasm; AML: acute myelogenous leukemia; PDAC: pancreatic ductal adenocarcinoma; MCN: mucinous cystic neoplasm.

To address this question of haploid insufficiency of p53 for tumor suppression, Venkatachalam et al. analyzed tumors from both $p53^{+/-}$ and $p53^{-/-}$ mice (12; Table 1) for the latency and tumor spectra. The survival of $p53^{+/-}$ mice (median, 70 weeks) was somewhere in between that of the $p53^{+/+}$, >100 weeks) and $p53^{-/-}$ mice (18 weeks). Of note, tumors that developed in $p53^{+/-}$ mice without loss of the remaining allele (lymphomas, sarcomas) occurred at later time points than those exhibiting LOH of the locus, suggesting that the wild type *p53* allele was partially effective for tumor suppression in $p53^{+/-}$ mice. A high proportion of tumors from the $p53^{+/-}$ mice retained a functional wild type *p53* allele. Thus a mere reduction in p53 levels was sufficient to promote tumorigenesis. Lynch and Milner determined both basal p53 mRNA and protein levels, and compared the p53 stress response in $p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$ isogenic clones derived from human colon adenocarcinoma cell line HCT116 [48]. Basal expression of p53 in $p53^{+/-}$ cells was only 25% in comparison to that in $p53^{+/+}$ cells, and this difference was maintained following oncogenic stress. The p53 stress responses were attenuated in $p53^{+/-}$ cells, in particular for $p21^{CIP1}$ upregulation, G1 arrest, and apoptosis [48]. This is the first study that demonstrated the molecular basis for haploinsufficiency of *p53*, which explains the attenuated tumor-suppressive phenotype observed in $p53^{+/-}$ mice and humans with LFS. In hematopoietic cells, p53-dependent cell cycle control, senescence, and apoptotic functions are actively involved in maintaining homeostasis under normal and stress conditions [49]. Whereas loss of p53 function promotes leukemia/lymphoma development, increased p53 activity results in myelodysplasia. Since genetic alterations in *TP53* is rather low in hematopoietic malignancies (10% - 20%, ref. 49), it is expected that p53 is functionally inactivated through other mechanisms e.g., *ARF* or *DMP1* deletion (see above).

Haploid insufficiency was also reported in the p53-binding protein 1, 53BP1 [50]. When DNA double-strand breaks (DSBs) occur, the cell cycle stage has a major influence on the choice of the repair pathway employed [51]. Specifically, non-homologous end joining is the predominant mechanism used in the G_1 phase of the cell cycle, while homologous recombination becomes fully activated in the S phase. Studies over the past 2 decades have revealed that the aberrant joining of replication-associated breaks leads to catastrophic genome rearrangements, revealing an important role of DNA break repair pathway choice in the preservation of genome integrity. 53BP1, a putative DNA-damage sensor that accumulates at sites of DSBs dependent on histone H2AX, and BRCA1, a well-known breast cancer tumor suppressor, are at the center of this choice [51].

Genomic profiling of human cancers has provided insights into the mutational landscape of genes that are involved in the response to radiation-induced DNA-damage. The sub-nuclear accumulation of proteins (i.e., foci) in the DNA-damage response (DDR), such as gamma-H2AX, 53BP1, or RAD51, has been studied as a substitute of treatment sensitivity [52]. Recent preclinical studies have demonstrated the predictive potential of DDR foci with clinically relevant end points, such as tumor control probability. This will yield functional insight that may complement or even supersede genomic information, thereby giving radiation oncologists unique opportunities to individualize cancer treatments in the near future [52].

Loss of a single *53BP1* allele compromised genomic stability and DSB repair, which could explain the susceptibility of *53BP1*$^{+/-}$ mice for tumorigenesis [50]. *53BP1* was heterozygously deleted in ~20% of human glioblastoma multiform specimens, and low 53BP1 expression was associated with worse prognosis. *53BP1* behaved as a haploinsufficient TSG in a mouse model of platelet-derived growth factor-induced gliomagenesis [53]. Since human glioma cell lines in which *53BP1* was robustly silenced by shRNA showed higher sensitivity to ionizing radiation, 53BP1 can be pharmacologically targeted in glioblastoma multiforme in combination with standard therapies [53].

DMP1 (DMTF1)

Dmp1, a cyclin *D* binding *m*yb-like *p*rotein *1* (also called Dmtf1), was originally isolated in a yeast two-hybrid screen of a murine T-lymphocyte library with cyclin D2 as bait [54, 55]. Dmp1 transactivates the myeloid differentiation marker *CD13* [128] and shows its activity as a tumor suppressor by directly binding to the *Arf* promoter to activate its gene expression and, thereby, induces Arf- and p53-dependent cell cycle arrest [56]. Recent studies show that it binds to endogenous cyclin D1 and transactivates both *p19*^{Arf} and *p16*^{Ink4a} [57, 58]. In *Arf*-null cells, Dmp1 physically interacts with p53 to induce senescence or apoptosis for tumor suppression [59; reviewed in 60]. The *Dmp1* promoter is efficiently activated by oncogenic Ras as well as by constitutively active MEK, ERK [61], but is repressed by E2Fs and NF-κB [62, 63]. Hence Dmp1 has been shown to be a key mediator between Ras-Raf-MEK-ERK mitogenic signaling and the Arf-p53 tumor suppressor pathway.

The activity of the Arf-53 pathway is significantly attenuated in *Dmp1*-deficient cells since those cells can easily give rise to immortalized cell lines that retain wild type $p19^{Arf}$ and functional p53 and are transformed by oncogenic Ras alone [3]. *Dmp1*-deficient mice are prone to tumor development [3, 4, Table 1]. Tumors induced by dimethylanthracene, *Eμ-Myc* [Figure 4A; ref. 4] or $K\text{-}Ras^{LA}$ [64] transgene were greatly accelerated in both $Dmp1^{+/-}$ and $Dmp1^{-/-}$ backgrounds, with no differences between groups lacking one or two *Dmp1* alleles [4; reviewed in 5]. Indeed, all tumors from $Dmp1^{+/-}$ mice retained and expressed the wild type *Dmp1* allele, and most expressed wild type Dmp1 mRNA and protein without inactivating mutation in both dimethylanthracene - treated mice and oncogene - transgenic cross, suggesting typical haploinsufficiency of Dmp1 in tumor suppression [4, 64, 65].

We recently characterized the signaling pathway between HER2/neu (Figure 4B) or cyclin D1 and Dmp1 using *MMTV-neu* or *MMTV-cyclin D1 (T286A)* mice as a model [57, 65]. Both Dmp1 and p53 were induced in pre-malignant hyperplastic lesions from *MMTV-neu** (mutant) mice, and mammary carcinogenesis was significantly accelerated in both $Dmp1^{+/-}$ and $Dmp1^{-/-}$ animals [65]. We also showed that constitutive expression of Dmp1α delayed HER2/neu-induced mammary tumorigenesis [58]. All of the mammary tumors from $Dmp1^{+/-}$; *neu** mice retained the wild type *Dmp1* allele (Figure 4B, left), and expressed the protein at levels less than 30% of those in $Dmp1^{wt}ND$; *neu** (i.e., wild type *neu* tumors without *Dmp1* deletion) [Figure 4B, right, ref. 65]. We also observed selective deletion of *Dmp1* in >50% of wild type *neu** mammary tumors (hemizygous *Dmp1* deletion: HD) while the involvement of *Arf, Mdm2*, or *p53* was rare [65]. Tumors from *Dmp1*-deficient mice showed significant downregulation of *Arf* and $p21^{Cip1}$, showing p53 inactivity and more aggressive phenotypes than tumors without *Dmp1* deletion [65]. Selective *Dmp1* deletion was also found in 21% of the *MMTV-D1* and *D1T286A* mammary tumors and the *Dmp1*-heterozygous status significantly accelerated mouse mammary tumorigenesis with reduced apoptosis and increased metastasis [57]. Thus our study shows the pivotal roles of *Dmp1* in HER2/neu-p53 and cyclin D1-$p16^{Ink4a}$-Rb signaling and mammary tumor development.

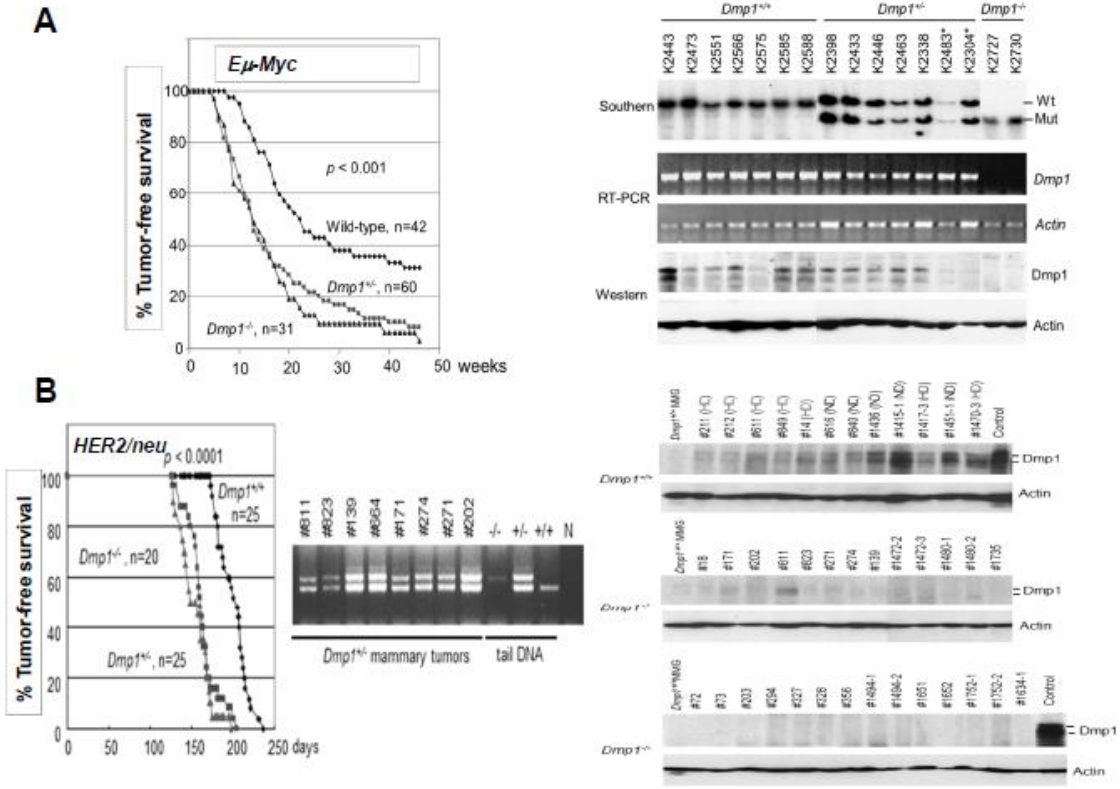

Figure 4. Haploid insufficiency of Dmp1 in tumor suppression.

A. Tumor development in *Eμ-Myc* TG mice [modified with permission from ref. 4].

Left: Tumor-free survival of *Eμ-Myc* TG animals of the indicated *Dmp1* genotypes. The disease-free survival was significantly shortened in both $Dmp1^{+/-}$ [box] and $Dmp1^{-/-}$ [triangle] mice (both $p < 0.001$ as compared to that of $Dmp1^{+/+}$*;Eμ-Myc* [diamond] mice) without significant difference between the two cohorts.

Right: Both *Dmp1* RNA and protein were detected by RT-PCR and immunoblotting, respectively, using β-actin as an internal control. Sequencing analyses showed no mutations within the DNA-binding domain for *Dmp1*. The Dmp1 protein was expressed in tumors from $Dmp1^{+/-}$ mice (~50% of that in $Dmp1^{+/+}$), but was undetectable in some of them (K2483* and K 2304*).

B. Tumor development in *MMTV-neu* mice [modified with permission from ref. 65].

Left: Tumor-free survival of *MMTV-neu* (mutant) mice of the indicated *Dmp1* genotypes. The ErbB2/neu gene is activated by point mutation in rat *neu* but an aberrant splicing in human breast cancer [127]. The disease-free survival was significantly shortened in both $Dmp1^{+/-}$ [box] and $Dmp1^{-/-}$ [triangle] in comparison to $Dmp1^{+/+}$ [diamond] mice ($p < 0.001$) without significant difference between the two cohorts. Genomic DNA PCR shows retention of the wild type allele in all mammary tumors from $Dmp1^{+/-}$ mice, showing haploinsufficiency.

Right: Western blotting analyses of *MMTV-neu* mammary tumors for Dmp1 using actin as an internal control. The Dmp1 protein expression was 2-16 folds higher in tumors from *Dmp1* wild type mice than normal mammary glands ($Dmp1^{+/+}$ MMG from a 15-week-old non-lactating female), reflecting the *Dmp1* promoter activation by HER2/neu. HD means spontaneous hemizygous deletion of the *Dmp1* locus in tumors from $Dmp1^{+/+}$*;MMTV-neu** mice. ND: no deletion. The protein expression levels were ~50% in HD tumors as compared with of those in ND tumors. The Dmp1 protein expression levels in $Dmp1^{+/-}$*;MMTV-neu** mice were ~25% of that in $Dmp1^{+/+}$*;MMTV-neu* ND* (no deletion) mice suggesting additional mechanism of destabilization of the protein in these tumors; however this is not important for tumor development since the survival curve for $Dmp1^{+/-}$; *MMTV-neu** mice was overlapping with that of $Dmp1^{-/-}$; *MMTV-neu** mice [65]. See ref. 132 for a general review for *MMTV*-driven mouse models.

Haplo-insufficient TSGs have been studied mainly in mice using gene-knockout mice than humans simply because it is easier to create artificial gene deletion models (homozygous or heterozygous deletion) in mice and conduct tumor development assays than analyzing numerous human cancer specimens where tissue availability can be an issue. Another hurdle to demonstrate the haploinsufficiency of a particular TSG in human cancers is a technical issue. The human *DMP1* (h*DMP1*; h*DMTF1*) gene is located on chromosome 7q21, a region often deleted in human breast/lung cancers and hematopoietic malignancies [66-68]. Bodner et al. reported that the *hDMP1* locus was hemizygously deleted in all of 7q- leukemia/lymphoma specimen regardless of the results for the detailed cytogenetic analysis [68]. We analyzed 51 human non-small cell lung carcinoma samples and found that LOH of h*DMP1* was present in ~35% of lung cancers, which happened in a mutually exclusive fashion with that of *INK4a/ARF* and/or *p53* in the same samples [64, 69]. LOH here means loss of one of the two genomic signals present in normal cells [64, 69]; thus our definition of LOH is different from loss of the mutant allele in tumor cells in classical TSGs. This raised the possibility that hemizygous h*DMP1* deletion might define a new disease entity with different response to therapy [70]. We then analyzed 110 pairs of normal and tumor tissues from breast cancer patients for LOH of h*DMP1*, *INK4a/ARF*, *p53* and gene amplification of *Hdm2* to study the frequency and prognostic value for each genetic alteration. LOH of the h*DMP1* locus was found in 42% of human breast carcinomas, while those of *INK4a/ARF* and *p53* were found in 20% and 34%, respectively. *Hdm2* amplification was found in 13% of the same sample, which was found independently of LOH for h*DMP1*. Again, LOH for the h*DMP1* locus was found in mutually exclusive fashion with that of *INK4a/ARF* and *p53*, and was associated with low Ki67 index and diploid karyotype [71]. Consistently, LOH for h*DMP1* was associated with luminal A category and longer relapse-free survival, while that of *p53* was associated with non-luminal A and shorter survival. Detailed LOH analyses with 10 independent primer sets showed that selective deletion of h*DMP1* was found in 79% of breast cancer [71] and 94% of lung cancer [64] indicating haploid insufficiency of h*DMP1* in suppressing human cancers.

The human *DMP1* locus encodes two other splice variants, namely DMP1β and γ, and our recent study indicates that the splice variant DMP1β protein is overexpressed in more than half of human breast cancers, and has oncogenic activity *in vivo* [72]. Although it blocks the activity of DMP1α [73], it has a p53-independent functions as well [72; reviewed in 74, 75]. Since

half of the DMP1β protein overexpression occurred simultaneously with LOH of the h*DMP1* locus in breast cancer [72], it was expected that these two events might synergize to lower the tumor-suppressive activities of DMP1α to less than 50% of those in wild type cells, which should be analyzed in detail. The function of the third transcript *DMP1γ* should also be analyzed *in vivo* through creation of transgenic mouse models.

ARF

CDKN2A (*INK4a/ARF*) is frequently disrupted in various types of human cancers, and germline mutations of this locus can confer susceptibility to melanoma and other tumors [76, 77]. Genetic disruption of $p19^{Arf}$ alone predisposes mice to tumorigenesis, demonstrating that *Arf* is a tumor-suppressor gene in mice [16]. *Arf* is a classical TSG in an *Eμ-Myc* B cell lymphoma model because tumor development is extremely accelerated in $Arf^{-/-}$ mice (7 weeks) in comparison to $Arf^{+/-}$ (11 weeks) or $Arf^{+/+}$ (30 weeks) mice, and because 80% of lymphomas from $Arf^{+/-}$ mice lost the wild type allele [78]. Krimpenfort et al. specifically mutated $p16^{Ink4a}$ and demonstrated that these mice, designated $Ink4a^{*/*}$, did not show a significant predisposition to spontaneous tumor formation within 17 months [79]. Murine embryonic fibroblasts (MEFs) derived from them proliferate normally, were mortal, and were not transformed by oncogenic *H-Ras* alone [79, 80]. The very mild phenotype of the $Ink4a^{*/*}$ mice indicated that the very strong phenotypes of the original $Ink4a/Arf^{\Delta 2,3}$ mice [79] were primarily due to loss of *Arf*. Of note, $Ink4a^{*/\Delta 2,3}$ mice that were deficient for *Ink4a* and heterozygous for *Arf* spontaneously developed a wide spectrum of tumors, including melanoma [79]. Those tumors expressed Arf demonstrating haploinsufficiency of *Arf* in the absence of *Ink4a* (Table 1). *Arf* haplo-insufficiency was also demonstrated in *Eμ-Myc* lymphomas where loss of one allele of *Arf* rescued protracted lymphoma development in $Mdm2^{+/-}$*; Eμ-Myc* TGs [81].

The inv(16) is one of the most frequent chromosomal translocations associated with acute myeloid leukemia (AML) and creates a chimeric fusion protein CBFβ/MYH11 [82]. The *ARF* tumor suppressor is transactivated by the AML1 protein (see below), suggesting that the inv(16) fusion protein may repress *ARF* expression. Moreno-Miralles et al. established a murine bone marrow transplant model of the inv(16) in which wild type, $Arf^{+/-}$, and $Arf^{-/-}$ bone marrow cells were engineered to express the inv(16) fusion protein [82].

Its expression was sufficient to induce a myelomonocytic AML even in wild type bone marrow, yet deletion of only a single *Arf* allele greatly accelerated the disease, indicating that *Arf* is haploinsufficient for the induction of AML in the presence of the inv(16) [82]. These reports indicate that even a classical tumor suppressor behaves like a haploinsufficient TSG when combined with other genetic alterations, which can happen frequently in human malignancies.

AML1:CBFβ

The core-binding factor (CBF) is a heterodimeric transcription factor complex that consists of 3 distinct DNA-binding CBFβ subunits (RUNX1, 2, and 3), and a common CBFβ subunit, which does not bind to DNA [83-87]. *RUNX1* was the first *CBF* gene to be isolated and has been known as *AML1, PEBPA2B,* or *CBFA2*. The binding affinity of AML1 subunit to the DNA promoter sequences is significantly increased by its association with CBFβ, protects AML1 subunit from proteolysis. Core-binding factor acute myeloid leukemia (AML) is cytogenetically defined by the presence of t(8;21)(q22;q22) or inv(16)(p13q22)/t(16;16)(p13;q22), producing *AML1-ETO* [83-86] and *CBFB/MYH11* [87] chimeric proteins, respectively, both contributing to leukemogenesis [85-87]. In both subtypes, the cytogenetic rearrangements disrupt genes that encode subunits of core-binding factor, a transcription factor that functions as an essential regulator of normal hematopoiesis. The t(3;21)(q26;q22) translocation, which is one of the consistent chromosomal abnormalities found in blastic crisis of chronic myelocytic leukemia, is thought to play an important role in the leukemic progression of CML to an acute blastic crisis phase. The *AML1* gene was also rearranged by the t(3;21)(q26;q22) translocation producing the AML1-Evi1 fusion protein [88], which leads to dysplastic hemopoiesis *in vivo* [89]. Lineage determinant Runx proteins organize and assemble multi-protein complexes at sites of transcription within the nucleus and regulate both RNA polymerase II- and I-mediated gene expression [90]. In addition, Runx proteins epigenetically control lineage determining transcriptional programs including: 1) architectural organization of macromolecular complexes in interphase,

2) regulation of gene expression through bookmarking during mitosis, and 3) microRNA-mediated translational control in the interphase nucleus. These mechanisms are compromised in the onset and progression of leukemias with altered RUNX proteins [90].

Null mutations in mouse *AML1* or *CBFβ* results in mid-gestational lethality with a complete lack of fetal liver hematopoiesis [91, 92]. Thus the AML1:CBFβ transcription factor complex is essential for hematopoiesis. Cai et al. examined the consequences of *AML1*-loss in hematopoietic stem cells (HSC) of the mouse embryo, and demonstrated an absolute requirement for *AML1* in functional HSCs [93]. Moreover, single allelic loss of *AML1* resulted in a dramatic change in the distribution of HSCs, leading to their early appearance in the aorta-gonad-mesonephros region and the yolk sac. The effect of *AML1* dosage on adult hematopoiesis was studied by comparing the hematopoietic systems of $AML1^{+/-}$ and $AML1^{+/+}$ mice [94]. Sun et al. reported that $AML1^{+/-}$ bone marrow had an increase in multilineage and lineage-restricted progenitors [94]. They also showed that even though $AML1^{+/-}$ mice had a decrease in the number of long-term repopulating hematopoietic stem cells, the engraftment levels were increased. These data demonstrate a dosage-dependent effect for AML1 in regulating the quantity of HSCs and their downstream lineage-committed hematopoietic progenitors [94].

Although $AML1^{+/-}$ mice are genetically comparable models with human FPD/AML, they do not develop spontaneous leukemia. To induce additional genetic alterations, retroviral insertional mutagenesis was performed with the use of *BXH2-TG* mice, which develop myeloid leukemia because of the random integration of retrovirus [95]. Heterozygous disruption of *AML1* in *BXH2-TG* mice resulted in a shortening of the latency period for developing leukemia. Moreover, the *c-Kit* gene that is frequently mutated in human RUNX leukemias was recurrently activated in *BXH2-TG;* $AML1^{+/-}$ mice, and a colony forming assay revealed synergism between monoallelic loss of *AML1* and c-Kit overexpression. Thus, the *BXH2-TG;* $AML1^{+/-}$ system is an excellent mouse model to study the mechanism of leukemogenesis in familial platelet disorder (FPD)/AML patients (Table 1).

Linggi et al. identified the $p14^{ARF}$ tumor suppressor as a direct transcriptional target of AML1-ETO, the product of t(8;21) frequently found in AML [84]. Of note, AML1 stimulated $p14^{ARF}$ transcription (Figure 3)

and induced phenotypes consistent with cellular senescence, which was antagonized by AML1-ETO [96]. This explains why p53 is not mutated in t(8;21)-containing leukemias.

FPD that predisposes to AML is an autosomal-dominant disease characterized by platelet defects, which tends to result in AML [97]. Mutational analysis of candidate genes on chromosome 21q revealed non-sense mutations or intragenic deletion of one allele of *AML1*. Analysis of blood cells from affected FPD/AML patients showed a decrease in megakaryocyte colony formation, demonstrating that *AML1* dosage affects megakaryopoiesis [98]. Hence haploinsufficiency of *AML1* is a pre-neoplastic condition that leads to acute leukemia in humans as well [98].

EGR1 AND 5Q-

The *Early growth response 1* (*Egr1*) gene encodes a zinc-finger transcription factor of 59,000 Daltons that activates transcription by binding to DNA as a monomer [99-101]. Depending on the cell type and the stimuli, EGR1 induces the expression of growth factors, growth factor receptors, proteins involved in the regulation of cell growth, differentiation, apoptosis, and extracellular matrix proteins. Because the consensus sequences for SP1- and EGR1-binding overlap, EGR1 often displaces SP1 from gene promoters; thus EGR1 competes with SP1 that is involved in the constitutive expression of housekeeping genes [so called ‘SP box’] and others.

The deletion of part of the long arm of chromosome 5 [del(5q)], is the most common chromosomal abnormality in primary myelodysplastic syndromes (MDS) and therapy-related myeloid neoplasms (t-MNs; ref. 21). Mouse models have implicated heterozygous loss of *APC*, *DIAPH1*, *EGR1*, and *NPM1* in the pathophysiology of MDS with del(5q) [102]. To study whether loss of *Egr1* is an initiating event in the pathogenesis of AML/MDS, *Egr1*-knockout mice were treated with ENU to induce cooperating mutations [103]. Both $Egr1^{+/-}$ and $Egr1^{-/-}$ mice treated with ENU developed T-cell lymphoma or a myeloproliferative disorder (MPD) at increased rates and with shorter latencies than those of wild type littermates (Table 1). Of note, biallelic inactivation of *Egr1* was not observed in MPDs in $Egr1^{+/-}$ mice, suggesting that haploinsufficiency for *Egr1* plays a role in murine leukemogenesis, and also in the development of AML/MDS with the alteration of chromosome 5.

It has been reported that loss of p53 activity, through mutation or deletion, is highly associated with t-MNs with a del(5q) [104-106]. Stoddart et al.

reported that loss of one copy of *Egr1* or *p53* in an $Apc^{del/+}$ background accelerated the development of a macrocytic anemia with monocytosis, early features of t-MN [106]. The development of $Apc^{del/+}$-induced anemia was significantly accelerated by treatment of mice with the alkylating agent ENU regardless of the genomic status of *Egr1* and *Tp53* demonstrating that the anemia was cell-extrinsic (i.e., caused by microenvironmental change). These data emphasized the synergistic role of cell intrinsic and extrinsic factors in the pathogenesis of t-MN, and raised awareness of the deleterious effects of cytotoxic therapy on the stromal microenvironment [106]. Stoddart et al. also showed that loss of *p53* in combination with mono-allelic loss of *Egr1* increased the rate of development of hematologic malignancies, but not overt myeloid leukemias [107]. Cell intrinsic loss of *p53* in $Egr1^{+/-}$; $Apc^{+/-}$ hematopoietic cells led to the development of AML in 17% of mice [107, Table 1]. Thus, loss of p53 activity in cooperation with *Egr1* and *Apc* haploinsufficiency creates an environment that predisposes to the development of AML.

TGFβ, TGFβR, AND SMAD4

Transforming growth factor-β (TGFβ) is a cytokine that controls cell proliferation and differentiation. It is involved in both inflammatory disorders and cancer. TGFβ acts as an anti-proliferative factor in normal epithelial cells at early stages of oncogenesis while it stimulates cell proliferation at advanced stages [108, 109]. Haploinsufficiency of TGFβ1 in tumor suppression was shown by the tumor predisposition of $TGF\beta^{+/-}$ mice [11]. However, because TGFβ is a secreted protein, its tumor-inhibitory effect depends on its expression in the whole animal, thus the mechanism for haploinsufficiency cannot be discussed within a single cell.

TGFβ dimers bind to a type II receptors which recruits and phosphorylates a type I receptor (Figure 5). The type I receptor then recruits and phosphorylates a receptor-regulated SMAD (R-SMAD; Smad 1, 2, 3, 5, 8/9). The R-SMAD then binds to the common SMAD:SMAD4 (also called DPC4) and forms a heterodimeric complex (Figure 5). This complex then enters the cell nucleus where it acts as a transcription factor for various genes to activate the mitogen-activated protein kinase 8 pathway, which triggers apoptosis.

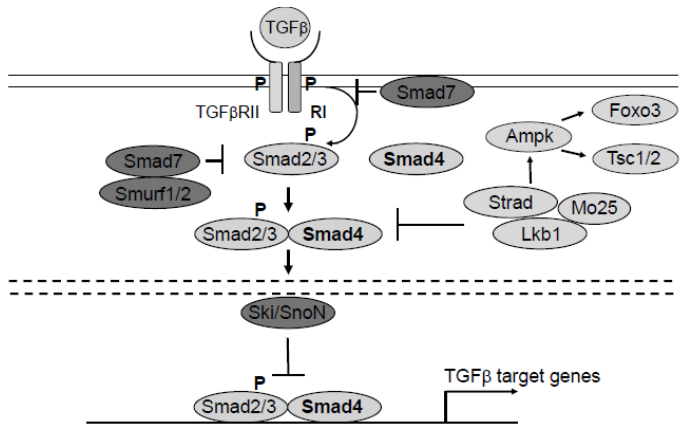

Figure 5. Signaling pathways involving TGFβ, Smad4, and Lkb1.

The solid lines represent the plasma membrane, and the discontinuous lines show the nuclear membrane. TGFβ binds to two distinct receptor types, known as type I and type II. Both type I and type II receptors contain serine/threonine kinase domains in their intracellular portions. The type II receptor kinases are constitutively active. When they bind to the ligand, they form hetero-tetrameric complexes composed of two molecules each of type I and type II receptors. In the hetero-tetrameric receptor I and II complexes, type II receptor kinases transphosphorylate the GS-domain of type I receptor, which, in turn, phosphorylate intracellular substrates. Smad proteins are major signaling molecules acting downstream of the serine/threonine kinase TGFβ receptors. Smads are classified into three subclasses, i.e., receptor-regulated Smads (R-Smads), common-partner Smad (co-Smad: Smad 4), and inhibitory Smads (I-Smads).

After activation of TGFβRI, the signal activates Smad2 and Smad3 proteins bound to the receptors, by phosphorylation of their C-terminal (SXS motif) residues. The next step is formation of a functional trimeric complex by phosphorylated R-Smad and Smad4, and then this complex is translocated to the nucleus, where it regulates the transcription of TGFβ1-dependent genes; thus Smads have the activity of transcription factors. The activity of TGFβ signaling pathway is regulated by a negative regulatory feedback loop mediated by I-Smads (Smads inhibitors): Smad 6 and 7. They are able to interact with membrane receptors by forming stable complex with activated TGFβRI, and thus impairing their interaction with the R-Smad (inhibition of their phosphorylation). Smad7 expression is induced by TGFβ, leading to inhibition of the cellular response to this cytokine. Smad7 has been shown to promote recruitment of E3 ubiquitin ligases, including Smad ubiquitin regulatory factors (Smurf1/2) into the receptor complex. Binding of Smad7 and Smurf to the receptor complex also results in competitive inhibition of Smad2/3 binding to TGFβRI. Direct Interaction of Ski with either Smad3 or Smad4 is necessary and sufficient for Ski-mediated repression of TGFβ signaling [133].

Lkb1 is a well-studied tumor suppressor kinase that regulates cell growth and polarity. It encodes a serine-threonine kinase that directly phosphorylates and activates AMPK, a central metabolic sensor [134]. Lkb1 is capable of phosphorylating Smad4 on its DNA-binding domain, inhibiting Smad4 from binding to either TGFβ- or bone morphogenetic protein-specific promoter sequences, thereby Smad4-dependent transcription [135]. Lkb1 activates AMPK and send signals to Foxo3, Tsc1/2, p53, fatty acid synthase, and other molecules, which enforces metabolic checkpoints for regulating cell growth and metabolism.

The TGFβ signaling pathway is frequently altered in colorectal cancer. Zheng et al. crossed *Tgfbr1*$^{+/-}$ mice with *Apc*$^{Min/+}$ mice, and showed that *Tgfbr1*$^{+/-}$; *Apc*$^{Min/+}$ mice developed twice as many intestinal tumors as *Tgfbr1*$^{+/+}$; *Apc*$^{Min/+}$ mice without losing the wild type *Tgfbr1* locus [110]. Decreased Smad2/3 phosphorylation, increased cyclin D1 expression, and cellular proliferation were observed in the colonic epithelium crypts of *Tgfbr1*$^{+/-}$; *Apc*$^{Min/+}$ mice. Thus *Tgfbr1* is haploinsufficient for tumor suppression and provides a molecular mechanism for colorectal cancer development in individuals with constitutively altered TGFβR1 expression [110, Table 1].

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the United States with a median survival of <6 months [111]. *KRAS* mutation is a hallmark of PDAC but remains an intractable pharmacologic target [112]. TGFβ signaling plays critical role in PDAC progression since *SMAD4* is deleted or mutated in 55% and the *TGFβR2* gene is altered in a smaller subset of human PDAC [113]. Pancreas-specific *Tgfβr2* knockout mice have been generated, alone or in combination of active Kras (Kras^{G12D}) expression driven by the pancreatic transcription factor 1a. Pancreas-specific Kras^{G12D} activation alone generated only intraepithelial neoplasia within 1 year [113]. Conversely, both *Tgfβr2*$^{-/-}$; *Kras*^{G12D} and *Tgfβr2*$^{+/-}$; *Kras*^{G12D} developed PDAC, which demonstrated a haploinsufficiency of TGFβ signaling in PDCA prevention (Table 1).

Smad4$^{+/-}$ mice begin to develop polyposis in the fundus at 6-12 months old, and in the duodenum and cecum in older animals. With increasing age, polyps in the antrum show sequential changes from hyperplasia to full adenocarcinoma. However, loss of the remaining wild type allele was detected only in later stages of tumor progression, suggesting that mono-allelic loss of *Smad4* is sufficient for tumor progression [114]. Alberici et al. [115] subsequently reported that haploinsufficiency of *Smad4* underlies tumor initiation in the gastrointestinal tract, which was sufficient to affect tumor progression both prior to and upon loss of *Apc* function (Table 1).

Oncogenic *Kras* initiates pancreatic tumorigenesis, while subsequent genetic events shape the resultant disease. Izeradjene et al. showed that concomitant expression of *Kras*^{G12D} and haploinsufficiency of the *Smad4/Dpc4* TSG induced a distinct class of pancreatic tumors, mucinous cystic neoplasms (MCNs), which developed into invasive ductal adenocarcinomas [116]. Progression of MCNs was accompanied by LOH of *Smad4* and mutation of either *p53* or *p16*^{Ink4a}. Thus, these distinct phenotypic

routes to invasive adenocarcinoma nevertheless share the same overall mutational spectra [116].

RESEARCH IN PROGRESS AND FUTURE DIRECTIONS

Haploinsufficiency of TSGs has been demonstrated mostly in mouse knockout models [18, 117], but analyses of human cancer specimen have also been performed in some cases. For instance, hemizygous loss of *hDMP1* was reported in 35-42% of lung and breast cancers [64, 71] with proof in mouse models [64, 65]. Since the wild type allele is retained without mutation or promoter hypermethylation in haploinsufficient TSGs, characterization of human cancer specimen for candidate haploinsufficient TSG, esp. for mutational analyses and the expression of the wild-type mRNA from the wild type locus, will be essential before an attempt to re-activate the wild type locus for novel cancer therapies.

The outstanding question for haploinsufficient TSG research is the molecular genetic mechanisms that explain the phenotype. In case of $p27^{Kip1}$, tumors from $p27^{Kip1+/-}$ mice express ~50% of the protein in comparison to $p27^{+/+}$ tumors, indicating that it is a typical haploinsufficient TSG [2]. In both *p27* and *Pten*, tumor development was accelerated in $TSG^{+/-}$ mice than in $TSG^{-/-}$ indicating that the tumor-prone phenotype was obligatory [9]. In case of *Eμ-Myc* tumors in *Dmp1* knockout mice, $Dmp1^{+/-}$ lymphomas expressed ~50% of the wild type protein, suggesting that it a typical case of haploid insufficiency [4]. However, it is not an obligatory haploinsufficiency since there was no significant difference in tumor-free survival between $Dmp1^{+/-}$ and $Dmp1^{-/-}$ mice [4; see 64, 65 for different models]. In case of *p53* knockout mice, it was shown that $p53^{+/-}$ cells express only 25% of wild type mRNA and protein, which could explain the acceleration of tumor development in $p53^{+/-}$ mice without losing the wild type allele [12, 48]. Likewise, the protein expression level in $Dmp1^{+/-}$*; MMTV-neu* tumors was ~25% as compared to those in $Dmp1^{+/+}$*; MMTV-neu* tumors [65]. Thus these are representative cases of 'one and a half' inactivation of the TSG loci. We have put both of these TSGs in the category of non-classical, haploinsufficient TSGs.

In the latter case of 'one and a half inactivation', it is reasonable to speculate that some unknown mechanisms may co-exist to lower, but they do not completely eliminate the expression of wild type tumor suppressive protein in $TSG^{+/-}$ tumors. Indeed, our recent study showed that the levels of *Dmp1* and *Arf* mRNAs in $Dmp1^{+/-}$ lungs were only 30 and 15%, respectively, of those in

Dmp1$^{+/+}$ tissues [118]. One possible mechanism for these findings is that the transcription for the *Dmp1* promoter is decreased in *Dmp1*$^{+/-}$ tissues through disruption of auto-regulation since the genomic locus has multiple Dmp1-binding consensus sequences. The second possibility for the decreased expression (i.e., less than 50%) of mRNA/protein in haploinsufficient *TSG*$^{+/-}$ tissues is accelerated degradation of the mRNA/protein, the former of which may be mediated by microRNAs that bind to the 3' non-coding region. In fact, bioinformatics analysis of haploinsufficient genes for variations in their 3′UTR showed that the occurrence of SNPs result in the creation of new binding sites for miRNAs [20]. Thus haploinsufficiency of TSGs can be driven by the cumulative effect of miRNAs, miRNA-binding-site polymorphisms, and miRNA polymorphisms. The third possibility is aberrant splicing of the locus that generates transcripts that block the activities the wild type TSG, which can co-exist with mono-allelic loss of the wild type allele locus of the TSG [72, 74]. In p63/p73, oncogenic splicing variants that lack the N-terminal sequences are often overexpressed in tumors and are associated with poor prognosis [119]. The mechanisms for degradation of haploinsufficient TSG protein products should be investigated since reversal for such process(es) can lead to a novel therapy of cancer.

This chapter focused on TSGs that are close to our research fields. We could not review other important haploinsufficient TSGs (e.g., *PTEN*, *LKB1*, *NF1*, *DOK2*, *NPM*, *RPS14*, or *NKX2-1/TTF-1*) due to limitations. Please refer to other reviews [9, 120-123] if you are interested in these molecules.

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REFERENCES

[1] Knudson, AG. Jr. Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci USA*, 1971, 68, 820-823.

[2] Fero, ML; Randel, E; Gurley, KE; Roberts, JM; Kemp, CJ. The murine gene p27Kip1 is haplo-insufficient for tumor suppression. *Nature*, 1998, 396, 177-180.

[3] Inoue, K; Wen, R; Rehg, JE; Adachi, M; Cleveland, JL; Roussel, MF; Sherr, CJ. Disruption of the ARF transcriptional activator DMP1 facilitates cell immortalization, ras transformation, and tumorigenesis. *Genes Dev*, 2000, 14, 1797-1809.

[4] Inoue, K; Zindy, F; Randle, DH; Rehg, JE; Sherr, CJ. Dmp1 is haplo-insufficient for tumor suppression and modifies the frequencies of Arf and p53 mutations in Myc-induced lymphomas. *Genes Dev*, 2001, 15, 2934-2939.

[5] Quon, KC, Berns A. Haplo-insufficiency? Let me count the ways. *Genes Dev*, 2001, 15, 2917-2921.

[6] Di Cristofano, A; Kotsi, P; Peng, YF; Cordon-Cardo, C; Elkon, KB; Pandolfi, PP. Impaired Fas response and autoimmunity in Pten+/− mice. *Science*, 1999, 285, 2122-2125.

[7] Di Cristofano, A; Pesce, B; Cordon-Cardo, C; Pandolfi, PP. Pten is essential for embryonic development and tumour suppression. *Nat Genet*, 1998, 19, 348-355.

[8] Gutmann, DH; Loehr, A; Zhang, Y; Kim, J; Henkemeyer, M; Cashen, A. Haploinsufficiency for the neurofibromatosis 1 (NF1) tumor suppressor results in increased astrocyte proliferation. *Oncogene*, 1999, 18, 4450-4459.

[9] Berger, AH; Pandolfi, PP. Haplo-insufficiency: a driving force in cancer. *J Pathol*, 2011, 223, 137-146.

[10] Chen, Z; Trotman, LC; Shaffer, D; Lin, HK; Dotan, ZA; Niki, M; Koutcher, JA; Scher, HI; Ludwig, T; Gerald, W; Cordon-Cardo, C; Pandolfi, PP. Crucial role of p53-dependent cellular senescence in suppression of *Pten*-deficient tumourigenesis. *Nature*, 2005, 436, 725-730.

[11] Tang, B; Böttinger, EP; Jakowlew, SB; Bagnall, KM; Mariano, J; Anver, MR; Letterio, JJ; Wakefield, LM. Transforming growth factor-B1 is a new form of tumor suppressor with true haploid insufficiency. *Nat Med*, 1998, 4, 802-807.

[12] Venkatachalam, S; Shi, YP; Jones, SN; Vogel, H; Bradley, A; Pinkel, D; Donehower, LA. Retention of wild type p53 in tumors from p53 heterozygous mice: reduction of p53 dosage can promote cancer formation. *EMBO J*, 1998, 17, 4657-4667.

[13] Varley, JM; Evans, DG; Birch, JM. Li-Fraumeni syndrome--a molecular and clinical review. *Br J Cancer*, 1997, 76, 1-14.

[14] Jacks, T; Remington, L; Williams, BO; Schmitt, EM; Halachmi, S; Bronson, RT; Weinberg, RA. Tumor spectrum analysis in p53-mutant mice. *Current Biol*, 1994, 4, 1-7.

[15] Serrano, M; Lee, H; Chin, L; Cordon-Cardo, C; Beach, D; DePinho, RA. Role of the INK4a locus in tumor suppression and cell mortality. *Cell*, 1996, 85, 27-37.

[16] Kamijo, T; Zindy, F; Roussel, MF; Quelle, DE; Downing, JR; Ashmun, RA; Grosveld, G; Sherr, CJ. Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. *Cell*, 1997, 91, 649-659.

[17] Sherr, CJ. Principles of tumor suppression. *Cell*, 2004, 116, 235-246.

[18] Taneja, P; Zhu, S; Maglic, D; Fry, EA; Kendig, RD; Inoue, K. Transgenic and knockout mice models to reveal the functions of tumour suppressor genes. *Clin Med Insights Oncol*, 2011, 5, 235-257.

[19] Alimonti, A; Carracedo, A; Clohessy, JG; Trotman, LC; Nardella, C; Egia, A; Salmena, L; Sampieri, K; Haveman, WJ; Brogi, E; Richardson, AL; Zhang, J; Pandolfi, PP. Subtle variations in Pten dose determine cancer susceptibility. *Nat Genet*, 2010, 42, 454-458.

[20] Manikandan, M; Raksha, G; Munirajan, AK. Haploinsufficiency of tumor suppressor genes is driven by the cumulative effect of microRNAs, microRNA binding site polymorphisms and microRNA polymorphisms: an insilico approach. *Cancer Inform*, 2012, 11, 157-171.

[21] Berger, AH; Knudson, AG; Pandolfi, PP. A continuum model for tumor suppression. *Nature*, 2011, 476, 163-169.

[22] Boultwood, J; Pellagatti, A; McKenzie, AN; Wainscoat, JS. Advances in the 5q- syndrome. *Blood*, 2010, 116, 5803-5811.

[23] Honda, H; Nagamachi, A; Inaba, T. -7/7q- syndrome in myeloid-lineage hematopoietic malignancies: attempts to understand this complex disease entity. *Oncogene*, 2015, 34, 2413-2425.

[24] Xue, W; Kitzing, T; Roessler, S; Zuber, J; Krasnitz, A; Schultz, N; Revill, K; Weissmueller, S; Rappaport, AR; Simon, J; Zhang, J; Luo, W; Hicks, J; Zender, L; Wang, XW; Powers, S; Wigler, M; Lowe, SW. A cluster of cooperating tumor-suppressor gene candidates in chromosomal deletions. *Proc Natl Acad Sci USA*, 2012, 109, 8212-8217.

[25] Sherr, CJ; Roberts, JM. Living with or without cyclins and cyclin-dependent kinases. *Genes Dev*, 2004, 18, 2699-2711.

[26] Malumbres, M; Barbacid, M. Cell cycle, CDKs and cancer: a changing paradigm. *Nat Rev Cancer*, 2009, 9, 153-166.

[27] Polyak, K; Kato, JY; Solomon, MJ; Sherr, CJ; Massague, J; Roberts, JM; Koff, A. p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes Dev*, 1994, 8, 9-22.

[28] Toyoshima, H; Hunter, T. p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. *Cell*, 1994, 78, 67-74.

[29] Sharma, SS; Pledger, WJ. The non-canonical functions of p27Kip1 in normal and tumor biology. *Cell Cycle*, 2016, 1-13.

[30] Cheng, M; Olivier, P; Diehl, JA; Fero, M; Roussel, MF; Roberts, JM; Sherr, CJ. The p21(Cip1) and p27(Kip1) CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts. *EMBO J*, 1999, 18, 1571-1583.

[31] Pietenpol, JA; Bohlander, SK; Sato, Y; Papadopoulos, N; Liu, B; Friedman, C; Trask, BJ; Roberts, JM; Kinzler, KW; Rowley, JD; Vogelstein, B. Assignment of the human p27Kip1 gene to 12p13 and its analysis in leukemias. *Cancer Res*, 1995, 55, 1206-1210.

[32] Muraoka, RS; Lenferink, AE; Law, B; Hamilton, E; Brantley, DM; Roebuck, LR; Arteaga, CL. ErbB2/Neu-induced, cyclin D1-dependent transformation is accelerated in p27-haploinsufficient mammary cells but impaired in p27-null cells. *Mol Cell Biol*, 2002, 22, 2204-2219.

[33] Le Toriellec, E; Despouy, G; Pierron, G; Gaye, N; Joiner, M; Bellanger, D; Vincent-Salomon, A; Stern, MH. Haploinsufficiency of CDKN1B contributes to leukemogenesis in T-cell prolymphocytic leukemia. *Blood*, 2008, 111, 2321-2328.

[34] Feurstein, S; Rücker, FG; Bullinger, L; Hofmann, W; Manukjan, G; Göhring, G; Lehmann, U; Heuser, M; Ganser, A; Döhner, K; Schlegelberger, B; Steinemann, D. Haploinsufficiency of ETV6 and CDKN1B in patients with acute myeloid leukemia and complex karyotype. *BMC Genomics*, 2014, 15, 784.

[35] Crona, J; Gustavsson, T; Norlén, O; Edfeldt, K; Åkerström, T; Westin, G; Hellman, P; Björklund, P; Stålberg, P. Somatic Mutations and Genetic Heterogeneity at the CDKN1B Locus in Small Intestinal Neuroendocrine Tumors. *Ann Surg Oncol*, 2015 Dec, 22 Suppl 3, S1428-35. doi: 10.1245/s10434-014-4351-9. Epub 2015 Jan 14.

[36] Ayrault, O; Zindy, F; Rehg, J; Sherr, CJ; Roussel, MF. Two tumor suppressors, p27Kip1 and patched-1, collaborate to prevent medulloblastoma. *Mol Cancer Res*, 2009, 7, 33-40.

[37] Diersch, S; Wenzel, P; Szameitat, M; Eser, P; Paul, MC; Seidler, B; Eser, S; Messer, M; Reichert, M; Pagel, P; Esposito, I; Schmid, RM; Saur, D; Schneider, G. Efemp1 and p27(Kip1) modulate responsiveness of pancreatic cancer cells towards a dual PI3K/mTOR inhibitor in preclinical models. *Oncotarget*, 2013, 4, 277-288.

[38] Vousden, KH; Prives, C. Blinded by the light: the growing complexity of p53. *Cell*, 2009, 137, 413-431.

[39] Menendez, D; Inga, A; Resnick, MA. The expanding universe of p53 targets. *Nat Rev Cancer*, 2009, 9, 724-737.

[40] Kruiswijk, F; Labuschagne, CF; Vousden, KH. p53 in survival, death and metabolic health: a lifeguard with a licence to kill. *Nat Rev Mol Cell Biol*, 2015, 16, 393-405.

[41] Muller, PA; Vousden, KH. Mutant p53 in cancer: new functions and therapeutic opportunities. *Cancer Cell*, 2014, 25, 304-317.

[42] Merino, D; Malkin, D. p53 and hereditary cancer. *Subcell Biochem*, 2014, 85, 1-16.

[43] Srivastava, S; Zou, ZQ; Pirollo, K; Blattner, W; Chang, EH. Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. *Nature*, 1990, 348, 747-749.

[44] Brosh, R; Rotter, V. When mutants gain new powers: news from the mutant p53 field. *Nat Rev Cancer*, 2009, 9, 701-713.

[45] Beckerman, R; Prives, C. Transcriptional regulation by p53. *Cold Spring Harb Perspect Biol*, 2010, 2, a000935.

[46] Lukashchuk, N; Vousden, KH. Ubiquitination and degradation of mutant p53. *Mol Cell Biol*, 2007, 27, 8284-8295.

[47] Davison, TS; Yin, P; Nie, E; Kay, C; Arrowsmith, CH. Characterization of the oligomerization defects of two p53 mutants found in families with Li-Fraumeni and Li-Fraumeni-like syndrome. *Oncogene*, 1998, 17, 651-656.

[48] Lynch, CJ; Milner, J. Loss of one p53 allele results in four-fold reduction of p53 mRNA and protein: a basis for p53 haploinsufficiency. *Oncogene*, 2006; 25: 3463-3470.

[49] Pant, V; Quintás-Cardama, A; Lozano, G. The p53 pathway in hematopoiesis: lessons from mouse models, implications for humans. *Blood*, 2012, 120, 5118-5127.

[50] Ward, IM; Difilippantonio, S; Minn, K; Mueller, MD; Molina, JR; Yu, X; Frisk, CS; Ried, T; Nussenzweig, A; Chen, J. 53BP1 cooperates with p53 and functions as a haploinsufficient tumor suppressor in mice. *Mol Cell Biol*, 2005, 25, 10079-10086.

[51] Daley, JM; Sung, P. 53BP1, BRCA1, and the choice between recombination and end joining at DNA double-strand breaks. *Mol Cell Biol*, 2014, 34, 1380-1388.

[52] Willers, H; Gheorghiu, L; Liu, Q; Efstathiou, JA; Wirth, LJ; Krause, M; von Neubeck, C. DNA damage response assessments in human tumor samples provide functional biomarkers of radiosensitivity. *Semin Radiat Oncol*, 2015, 25, 237-250.

[53] Squatrito, M; Vanoli, F; Schultz, N; Jasin, M; Holland, EC. 53BP1 is a haploinsufficient tumor suppressor and protects cells from radiation response in glioma. *Cancer Res*, 2012, 72, 5250-526.

[54] Inoue, K; Sherr, CJ. Gene expression and cell cycle arrest mediated by transcription factor DMP1 is antagonized by D-type cyclins through a cyclin-dependent-kinase-independent mechanism. *Mol Cell Biol*, 1998, 18, 1590-1600.

[55] Inoue, K; Mallakin, A; Frazier, DP. Dmp1 and tumor suppression. *Oncogene*, 2007, 26, 4329-4335.

[56] Inoue, K; Roussel, MF; Sherr, CJ. Induction of ARF tumor suppressor gene expression and cell cycle arrest by transcription factor DMP1. *Proc Natl Acad Sci USA*, 1999, 96, 3993-3998.

[57] Zhu, S; Mott, RT; Fry, EA; Taneja, P; Kulik, G; Sui, G; Inoue, K. Cooperation between cyclin D1 expression and Dmp1-loss in breast cancer. *Am J Pathol*, 2013, 183, 1339-1350.

[58] Fry, EA; Taneja, P; Maglic, D; Zhu, S; Sui, G; Inoue, K. Dmp1α inhibits HER2/neu-induced mammary tumorigenesis. *PLoS One*, 2013, 8, e77870.

[59] Frazier, DP; Kendig, RD; Kai, F; Maglic, D; Sugiyama, T; Taneja, P; Morgan, RL; Fry, EA; Lagedrost, SJ; Sui, G; Inoue, K. Dmp1 physically interacts with p53 and positively regulates p53's stabilization, nuclear localization, and function. *Cancer Res*, 2012, 72, 1740-1750.

[60] Inoue, K; Fry, EA; Frazier, DP. Transcription factors that interact with p53 and Mdm2. *Int J Cancer*, 2016, 138, 1577-1585.

[61] Sreeramaneni, R; Chaudhry, A; McMahon, M; Sherr, CJ; Inoue, K. Ras-Raf-Arf signaling critically depends on the Dmp1 transcription factor. *Mol Cell Biol*, 2005, 25, 220-232.

[62] Mallakin, A; Taneja, P; Matise, LA; Willingham, MC; Inoue, K. Expression of Dmp1 in specific differentiated, nonproliferating cells and its repression by E2Fs. *Oncogene*, 2006, 25, 7703-7713.

[63] Taneja, P; Mallakin, A; Matise, LA; Frazier, DP; Choudhary, M; Inoue, K. Repression of Dmp1 and Arf promoter by anthracyclins: critical roles of the NF-κB subunit p65. *Oncogene*, 2007, 26, 7457-7466.

[64] Mallakin, A; Sugiyama, T; Taneja, P; Matise, LA; Frazier, DP; Choudhary, M; Hawkins, GA; D'Agostino, RB; Jr. Willingham, MC; Inoue, K. Mutually exclusive inactivation of DMP1 and ARF/p53 in lung cancer. *Cancer Cell*, 2007, 12, 381-394.

[65] Taneja, P; Maglic, D; Kai, F; Sugiyama, T; Kendig, RD; Frazier, DP; Willingham, MC; Inoue, K. Critical role of Dmp1 in HER2/neu-p53 signaling and breast carcinogenesis. *Cancer Res*, 2010, 70, 9084-9094.

[66] Cornelisse, CJ. Loss of heterozygosity, chromosome 7q, and breast cancer. *Lancet*, 1992, 339, 1423-1424.

[67] Kristjansson, AK. Loss of heterozygosity at chromosome 7q in human breast cancer: association with clinical variables. *Anticancer Res*, 1997, 17, 93-98.

[68] Bodner, SM; Naeve, CW; Rakestraw, KM; Jones, BG; Valentine, VA; Valentine, MB; Luthardt, FW; Willman, CL; Raimondi, SC; Downing, JR; Roussel, MF; Sherr, CJ; Look, AT. Cloning and chromosomal localization of the gene encoding human cyclin D-binding Myb-like protein (hDMP1). *Gene*, 1999, 229, 223-228.

[69] Inoue, K; Sugiyama, T; Taneja, P; Morgan, RL; Frazier, DP. Emerging roles of DMP1 in lung cancer. *Cancer Res*, 2008, 68, 4487-4490.

[70] Sugiyama, T; Frazier, DP; Taneja, P; Morgan, RL; Willingham, MC; Inoue, K. The role of Dmp1 and its future in lung cancer diagnostics. *Expert Rev Mol Diagn*, 2008, 8, 435-448.

[71] Maglic, D; Zhu, S; Fry, EA; Taneja, P; Kai, F; Kendig, RD; Sugiyama, T; Miller, LD; Willingham, MC; Inoue, K. Prognostic value of the hDMP1-ARF-Hdm2-p53 pathway in breast cancer. *Oncogene*, 2013, 32, 4120-4129.

[72] Maglic, D; Stovall, DB; Cline, JM; Fry, EA; Mallakin, A; Taneja, P; Caudell, DL; Willingham, MC; Sui, G; Inoue, K. DMP1β, a splice isoform of the tumor suppressor DMP1 locus, induces proliferation and progression of breast cancer. *J Pathol*, 2015, 236, 90-102.

[73] Tschan, MP; Federzoni, EA; Haimovici, A; Britschgi, C; Moser, BA; Jin, J; Reddy, VA; Sheeter, DA; Fischer, KM; Sun, P; Torbett, BE. Human DMTF1β antagonizes DMTF1α regulation of the $p14^{ARF}$ tumor suppressor and promotes cellular proliferation. *Biochim Biophys Acta*, 2015, 1849, 1198-1208.

[74] Inoue, K; Fry, EA. Aberrant splicing of the DMP1-INK4a/ARF-MDM2-p53 pathway in cancer. *Int J Cancer*, 2016, Jan 23. doi: 10.1002/ijc.30003. [Epub ahead of print] PMID: 26802432.

[75] Inoue, K; Fry, EA. Novel biomarkers for breast cancer. *Biomark Cancer*, 2016, 8, 1-18.

[76] Ozenne, P; Eymin, B; Brambilla, E; Gazzeri, S. The ARF tumor suppressor: structure, functions and status in cancer. *Int J Cancer*, 2010, 127, 2239-2247.

[77] Maggi, LB; Jr. Winkeler, CL; Miceli, AP; Apicelli, AJ; Brady, SN; Kuchenreuther, MJ; Weber, JD. ARF tumor suppression in the nucleolus. *Biochim Biophys Acta*, 2014, 1842, 831-839.

[78] Eischen, CM; Weber, JD; Roussel, MF; Sherr, CJ; Cleveland, JL. Disruption of the ARF-Mdm2-p53 tumor suppressor pathway in Myc-induced lymphomagenesis. *Genes Dev*, 1999, 13, 2658-2669.

[79] Krimpenfort, P; Quon, KC; Mooi, WJ; Loonstra, A; Berns, A. Loss of p16INK4a confers susceptibility to metastatic melanoma in mice. *Nature*, 2001, 413, 83-86.

[80] Sharpless, NE; Bardeesy, N; Lee, KH; Carrasco, D; Castrillon, DH; Aguirre, AJ; Wu, EA; Horner, JW; DePinho, RA. Loss of p16Ink4a with retention of p19Arf predisposes mice to tumorigenesis. *Nature*, 2001, 413, 86-91.

[81] Eischen, CM; Alt, JR; Wang, P. Loss of one allele of ARF rescues Mdm2 haploinsufficiency effects on apoptosis and lymphoma development. *Oncogene*, 2004, 23, 8931-8940.

[82] Moreno-Miralles, I; Pan, L; Keates-Baleeiro, J; Durst-Goodwin, K; Yang, C; Kim, HG; Thompson, MA; Klug, CA; Cleveland, JL; Hiebert, SW. The inv(16) cooperates with ARF haploinsufficiency to induce acute myeloid leukemia. *J Biol Chem*, 2005, 280, 40097-40103.

[83] Sangle, NA; Perkins, SL. Core-binding factor acute myeloid leukemia. *Arch Pathol Lab Med*, 2011, 135, 1504-1509.

[84] Ichikawa, M; Yoshimi, A; Nakagawa, M; Nishimoto, N; Watanabe-Okochi, N; Kurokawa, M. A role for RUNX1 in hematopoiesis and myeloid leukemia. *Int J Hematol*, 2013, 97, 726-734.

[85] Nakazawa, T; Harada, Y; Tatsumi, N; Tsuboi, A; Kawakami, M; Oka, Y; Oji, Y; Aozasa, K; Kawase, I; Sugiyama, H. AML1-ETO rapidly induces acute myeloblastic leukemia in cooperation with the Wilms tumor gene, WT1. *Blood*, 2006, 107, 3303-3312.

[86] Yan, M; Kanbe, E; Peterson, LF; Boyapati, A; Miao, Y; Wang, Y; Chen, IM; Chen, Z; Rowley, JD; Willman, CL; Zhang, DE. A previously

unidentified alternatively spliced isoform of t(8;21) transcript promotes leukemogenesis. *Nat Med*, 2006, 12, 945-949.

[87] Lukasik, SM; Zhang, L; Corpora, T; Tomanicek, S; Li, Y; Kundu, M; Hartman, K; Liu, PP; Laue, TM; Biltonen, RL; Speck, NA; Bushweller, JH. Altered affinity of CBF beta-SMMHC for Runx1 explains its role in leukemogenesis. *Nat Struct Biol*, 2002, 9, 674-679.

[88] Mitani, K; Ogawa, S; Tanaka, T; Miyoshi, H; Kurokawa, M; Mano, H; Yazaki, Y; Ohki, M; Hirai, H. Generation of the AML1-EVI-1 fusion gene in the t(3;21)(q26;q22) causes blastic crisis in chronic myelocytic leukemia. *EMBO J*, 1994, 13, 504-510.

[89] Maki, K; Yamagata, T; Asai, T; Yamazaki, I; Oda, H; Hirai, H; Mitani, K. Dysplastic definitive hematopoiesis in AML1/EVI1 knock-in embryos. *Blood*, 2005, 106, 2147-2155.

[90] Zaidi, SK; Trombly, DJ; Dowdy, CR; Lian, JB; Stein, JL; van Wijnen, AJ; Stein, GS. Epigenetic mechanisms in leukemia. *Adv Biol Regul*, 2012, 52, 369-376.

[91] Okuda, T; van Deursen, J; Hiebert, SW; Grosveld, G; Downing, JR. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell*, 1996, 84, 321-330.

[92] Sasaki, K; Yagi, H; Bronson, RT; Tominaga, K; Matsunashi, T; Deguchi, K; Tani, Y; Kishimoto, T; Komori, T. Absence of fetal liver hematopoiesis in mice deficient in transcriptional coactivator core binding factor beta. *Proc Natl Acad Sci USA*, 93, 12359-12363.

[93] Cai, Z; de Bruijn, M; Ma, X; Dortland, B; Luteijn, T; Downing, RJ; Dzierzak, E. Haploinsufficiency of AML1 affects the temporal and spatial generation of hematopoietic stem cells in the mouse embryo. *Immunity*, 2000, 13, 423-431.

[94] Sun, W; Downing, JR. Haploinsufficiency of AML1 results in a decrease in the number of LTR-HSCs while simultaneously inducing an increase in more mature progenitors. *Blood*, 2004, 104, 3565-3672.

[95] Yamashita, N; Osato, M; Huang, L; Yanagida, M; Kogan, SC; Iwasaki, M; Nakamura, T; Shigesada, K; Asou, N; Ito, Y. Haploinsufficiency of Runx1/AML1 promotes myeloid features and leukaemogenesis in BXH2 mice. *Br J Haematol*, 2005, 131, 495-507.

[96] Linggi, B; Müller-Tidow, C; van de Locht, L; Hu, M; Nip, J; Serve, H; Berdel, WE; van der Reijden, B; Quelle, DE; Rowley, JD; Cleveland, J; Jansen, JH; Pandolfi, PP; Hiebert, SW. The t(8;21) fusion protein, AML1 ETO, specifically represses the transcription of the p14(ARF)

tumor suppressor in acute myeloid leukemia. *Nat Med*, 2002, 8, 743-750.

[97] Minelli, A; Maserati, E; Rossi, G; Bernardo, ME; De Stefano, P; Cecchini, MP; Valli, R; Albano, V; Pierani, P; Leszl, A; Sainati, L; Lo Curto, F; Danesino, C; Locatelli, F; Pasquali, F. Familial platelet disorder with propensity to acute myelogenous leukemia: genetic heterogeneity and progression to leukemia via acquisition of clonal chromosome anomalies. *Genes Chromosome Canc*, 2004, 40, 165-171.

[98] Song, WJ; Sullivan, MG; Legare, RD; Hutchings, S; Tan, X; Kufrin, D; Ratajczak, J; Resende, IC; Haworth, C; Hock, R; Loh, M; Felix, C; Roy, DC; Busque, L; Kurnit, D; Willman, C; Gewirtz, AM; Speck, NA; Bushweller, JH; Li, FP; Gardiner, K; Poncz, M; Maris, JM; Gilliland, DG. Haploinsufficiency of CBFA2 causes familial thrombocytopenia with propensity to develop acute myelogenous leukemia. *Nat Genet*, 1999, 23, 166-175.

[99] Baron, V; Adamson, ED; Calogero, A; Ragona, G; Mercola, D. The transcription factor Egr1 is a direct regulator of multiple tumor suppressors including TGFbeta1, PTEN, p53, and fibronectin. *Cancer Gene Ther*, 2006, 13, 115-124.

[100] Pagel, JI; Deindl, E. Early growth response 1--a transcription factor in the crossfire of signal transduction cascades. *Indian J Biochem Biophys*, 2011, 48, 226-235.

[101] Zwang, Y; Oren, M; Yarden, Y. Consistency test of the cell cycle: roles for p53 and EGR1. *Cancer Res*, 2012, 72, 1051-1054.

[102] Ebert, BL. Molecular dissection of the 5q deletion in myelodysplastic syndrome. *Sem Oncol*, 2011, 38, 621-626.

[103] Joslin, JM; Fernald, AA; Tennant, TR; Davis, EM; Kogan, SC; Anastasi, J; Crispino, JD; Le Beau, MM. Haploinsufficiency of EGR1, a candidate gene in the del(5q), leads to the development of myeloid disorders. *Blood*, 2007, 110, 719-726.

[104] Cleven, AH; Nardi, V; Ok, CY; Goswami, M; Dal Cin, P; Zheng, Z; Iafrate, AJ; Abdul Hamid, MA; Wang, SA; Hasserjian, RP. High p53 protein expression in therapy-related myeloid neoplasms is associated with adverse karyotype and poor outcome. *Mod Pathol*, 2015, 28, 552-563.

[105] Wang, SA; Bueso-Ramos, CE. MLL gene amplification in acute myeloid leukemia and myelodysplastic syndromes is associated with characteristic clinicopathological findings and TP53 gene mutation. *Hum Pathol*, 2015, 46, 65-73.

[106] Stoddart, A; Wang, J; Fernald, AA; Karrison, T; Anastasi, J; Le Beau, MM. Cell intrinsic and extrinsic factors synergize in mice with haploinsufficiency for Tp53, and two human del(5q) genes, Egr1 and Apc. *Blood*, 2014, 123, 228-238.

[107] Stoddart, A, Fernald, AA; Wang, J; Davis, EM; Karrison, T; Anastasi, J; Le Beau, MM. Haploinsufficiency of del(5q) genes, Egr1 and Apc, cooperate with Tp53 loss to induce acute myeloid leukemia in mice. *Blood*, 2014, 123, 1069-1078.

[108] Inman, GJ. Switching TGFβ from a tumor suppressor to a tumor promoter. *Curr Opin Genet Dev Cold Spring Harb Perspect Biol*, 2011, 21, 93-99.

[109] Principe, DR; Doll, JA; Bauer, J; Jung, B; Munshi, HG; Bartholin, L; Pasche, B; Lee, C; Grippo, PJ. TGF-β: Duality of function between tumor prevention and carcinogenesis. *J Natl Cancer Inst*, 2014, 106, djt369.

[110] Zeng, Q; Phukan, S; Xu, Y; Sadim, M; Rosman, DS; Pennison, M; Liao, J; Yang, GY; Huang, CC; Valle, L; Di Cristofano, A; de la Chapelle, A; Pasche, B. Tgfbr1 haploinsufficiency is a potent modifier of colorectal cancer development. *Cancer Res*, 2009, 69, 678-686.

[111] Ying, H; Dey, P; Yao, W; Kimmelman, AC; Draetta, GF; Maitra, A; DePinho, RA. Genetics and biology of pancreatic ductal adenocarcinoma. *Genes Dev*, 2016, 30, 355-385.

[112] Collisson, EA. A central role for RAF→MEK→ERK signaling in the genesis of pancreatic ductal adenocarcinoma. *Cancer Discov*, 2012, 2, 685-693.

[113] Ijichi, H; Chytil, A; Gorska, AE; Aakre, ME; Fujitani, Y; Fujitani, S; Wright, CV; Moses, HL. Aggressive pancreatic ductal adenocarcinoma in mice caused by pancreas-specific blockade of transforming growth factor-beta signaling in cooperation with active Kras expression. *Genes Dev*, 2006, 20, 3147-3160.

[114] Xu, X; Brodie, SG; Yang, X; Im, YH; Parks, WT; Chen, L; Zhou, YX; Weinstein, M; Kim, SJ; Deng, CX. Haploid loss of the tumor suppressor Smad4/Dpc4 initiates gastric polyposis and cancer in mice. *Oncogene*, 2000, 19, 1868-1874.

[115] Alberici, P; Jagmohan-Changur, S; De Pater, E; Van Der Valk, M; Smits, R; Hohenstein, P; Fodde, R. Smad4 haploinsufficiency in mouse models for intestinal cancer. *Oncogene*, 2006, 25, 1841-1851.

[116] Izeradjene, K; Combs, C; Best, M; Gopinathan, A; Wagner, A; Grady, WM; Deng, CX; Hruban, RH; Adsay, NV; Tuveson, DA; Hingorani,

SR. Kras(G12D) and Smad4/Dpc4 haploinsufficiency cooperate to induce mucinous cystic neoplasms and invasive adenocarcinoma of the pancreas. *Cancer Cell*, 2007, 11, 229-243.

[117] Inoue, K; Fry, EA; Taneja, P. Recent progress in mouse models for tumor suppressor genes and its implications in human cancer. *Clin Med Insights Oncol*, 2013, 7, 103-122.

[118] Mallakin, A; Sugiyama, T; Kai, F; Taneja, P; Kendig, RD; Frazier, DP; Maglic, D; Matise, LA; Willingham, MC; Inoue, K. The Arf-inducing transcription factor Dmp1 encodes transcriptional activator of amphiregulin, thrombospondin-1, JunB and Egr1. *Int J Cancer*, 2010, 126, 1403-1416.

[119] Inoue, K; Fry, EA. Alterations of p63 and p73 in human cancers. *Subcell Biochem*, 2014, 85, 17-40.

[120] Salmena, L; Carracedo, A; Pandolfi, PP. Tenets of PTEN tumour suppression. *Cell*, 2008, 133, 403-414.

[121] Larizza, L; Gervasini, C; Natacci, F; Riva, P. Developmental abnormalities and cancer predisposition in neurofibromatosis type 1. *Curr Mol Med*, 2009, 9, 634-653.

[122] Yamaguchi, T; Hosono, Y; Yanagisawa, K; Takahashi, T. NKX2-1/TTF-1: an enigmatic oncogene that functions as a double edged sword for cancer cell survival and progression. *Cancer Cell*, 2013, 23, 713-723.

[123] Pellagatti, A; Boultwood, J. Recent Advances in the 5q- Syndrome. *Mediterr J Hematol Infect Dis*, 2015, 7, e2015037.

[124] Jacks, T; Fazeli, A; Schmitt, EM; Bronson, RT; Godell, MA; Weinberg, RA. Effects of Rb mutation in the mouse. *Nature*, 2002, 359, 295-300.

[125] David, CJ; Manley, JL. Alternative pre-mRNA splicing regulation in cancer: pathways and programs unhinged. *Genes Dev*, 2010, 24, 2343-2364.

[126] Chen, J; Weiss, WA. Alternative splicing in cancer: implications for biology and therapy. *Oncogene*, 2015, 34, 1-14.

[127] Inoue, K; Fry, EA. Aberrant splicing of estrogen receptor, HER2, and CD44 in breast cancer. *Genet Epigenet*, 2015, 7, 19-32.

[128] Inoue, K; Sherr, CJ; Shapiro, LH. Regulation of the CD13/ aminopeptidase N gene by DMP1, a transcription factor antagonized by D-type cyclins. *J Biol Chem*, 1998, 273, 29188-21994.

[129] Inoue, K; Fry, EA. Aberrant expression of Cyclin D1 in cancer. *Signal Transduction Insights*, 2015, 4, 1-13.

[130] Taneja, P; Frazier, DP; Sugiyama, T; Lagedrost, SJ; Inoue, K. Control of cellular physiology by transcription factors E2F and their roles in

'Carcinogenesis in Control of Cellular Physiology by E2F transcription factors'. Editor: Ken-ichi Yoshida. Research Signpost, Trivandrum, Kerala, India. Chapter 10, 179-197, 2008.

[131] Chen, HZ; Tsai, SY; Leone, G. Emerging roles of E2Fs in cancer: an exit from cell cycle control. *Nat Rev Cancer*, 2009, 9, 785-797.

[132] Taneja, P; Frazier, DP; Kendig, RD; Maglic, D; Sugiyama, T; Kai, F; Taneja, NK; Inoue, K. MMTV mouse models and the diagnostic values of MMTV-like sequences in human breast cancer. *Expert Rev Mol Diagn*, 2009, 9, 423-440.

[133] Ueki, N; Hayman, MJ. Direct interaction of Ski with either Smad3 or Smad4 is necessary and sufficient for Ski-mediated repression of transforming growth factor-beta signaling. *J Biol Chem*, 2003, 278, 32489-32492.

[134] Shackelford, DB1; Shaw, RJ. The LKB1-AMPK pathway: metabolism and growth control in tumour suppression. *Nat Rev Cancer*, 2009, 9, 563-575.

[135] Morén, A; Raja, E; Heldin, CH; Moustakas, A. Negative regulation of TGFβ signaling by the kinase LKB1 and the scaffolding protein LIP1. *J Biol Chem*, 2011, 286, 341-353.

[136] Kamijo, T; Bodner, S; van de Kamp, E; Randle, DH; Sherr, CJ. Tumor spectrum in ARF-deficient mice. *Cancer Res*, 1999, 59, 2217-2222.

BIOGRAPHICAL SKETCH

Kazushi Inoue, M.D., Ph.D.

Affiliation: Wake Forest University Health Sciences

Education: 1984: Gifu University, M.D.
1990: The University of Tokyo, Ph.D.

Address: 205 Crowne Club Drive #5, Winston-Salem, NC 27104 USA

Research and Professional Experience:

1986-1990: Cancer Genetics at the Institute of Medical Science, the University of Tokyo

1991-1996: Clinical Research Fellow (Hematology/Oncology) at the Dept. of Medicine III, Osaka University School of Medicine

1996-2002: Cancer Research at the Dept. of Tumor Cell Biology, St. Jude Children's Research Hospital
2002-: Cancer Research at the Dept. of Pathology, Wake Forest University Health Sciences

Professional Appointments:

April 1, 1996: Associate Investigator, Dept. of Tumor Cell Biology, St. Jude Children's Research Hospital
September 1, 2002: Assistant Professor of Pathology, Wake Forest University Health Sciences
July 1, 2010: Associate Professor of Pathology, Wake Forest University Health Sciences

Publications in the last 3 years:

Primary Articles.

1. Maglic D, Zhu S, Fry EA, Taneja P, Kai F, Kendig RD, Sugiyama T, Miller LD, Willingham MC, and Inoue K. Prognostic value of the hDMP1-ARF-Hdm2-p53 pathway in breast cancer. *Oncogene* 2013 Aug 29; 32(35): 4120-9.
2. Zhu S, Mott RT, Fry EA, Taneja, P, Kulik G, Sui G, and Inoue K. Cooperation between cyclin D1 expression and *Dmp1*-loss in breast cancer. *Am J Pathol* 2013 Oct; 183(4): 1339-50. doi: 10.1016/j.ajpath.2013.06.027. Epub 2013 Aug 11.
3. Fry EA, Taneja P, Maglic D, Zhu S, Sui G, and Inoue K. Dmp1α inhibits HER2/neu-induced mammary tumorigenesis. *PLoS One* 2013 Oct 29; 8(10): e77870. doi: 10.1371/journal.pone.0077870.
4. Maglic D, Stovall DB, Cline JM, Fry EA, Mallakin A, Taneja P, Caudell DL, Willingham MC, Sui G, and Inoue K. DMP1β, a splice isoform of the tumor suppressor *DMP1* locus, induces proliferation and progression of breast cancer. *J Pathol* 2015 May; 236(1): 90-102. doi: 10.1002/path.4504.

Review Articles.

1. Inoue K, Fry EA, and Taneja P. Recent progress in mouse models for tumor suppressor genes and its implications in human cancer. *Clin Med Insight Oncol* 7:103-22, 2013.
2. Inoue K, Fry EA, and Frazier DP. Transcription factors that interact with p53 and Mdm2. *Int J Cancer* 2015 Jul 1. doi: 10.1002/ijc.29663. [Epub ahead of print]
3. Inoue K and Fry EA. Aberrant splicing of estrogen receptor, HER2, and CD44 in breast cancer. *Genet Epigenet* 7:19-32, 2015.
4. Inoue K and Fry EA. Aberrant splicing of the DMP1-INK4a/ARF-MDM2-p53 pathway in cancer. *Int J Cancer* 2016, Jan 23. doi: 10.1002/ijc.30003. [Epub ahead of print]
5. Inoue K and Fry EA. Novel biomarkers for breast cancer. *Biomark Cancer* 8:1-18, 2016.

Book Chapter

1. Inoue K and Fry EA. Alterations of p63 and p73 in human cancers in 'Mutant p53 and MDM2 in cancer'. Editors: Swati Palit Deb and Sumitra Deb. Springer, NY. Chapter 2: 17-40, 2014.
Subcell Biochem 85:17-40, 2014.

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Chapter 7

ANIMAL MODELS OF RESPIRATORY DISTRESS IN NEONATES

Daniela Mokra*, PhD
Biomedical Center Martin and Department of Physiology,
Jessenius Faculty of Medicine in Martin,
Comenius University in Bratislava,
Martin, Slovakia

ABSTRACT

A respiratory distress in neonates developed from various reasons is characterized by a reduced ability or an inability to supply gas exchange. This situation may originate from a prematurity and an insufficient synthesis of a pulmonary surfactant, i.e., respiratory distress syndrome (RDS) in preterm neonates. Another reason is represented by a secondary inactivation of the surfactant in the term neonates, e.g., from bacterial endotoxins in pneumonia or from aspiration of a meconium. To find out an appropriate treatment, a special *in vivo* testing in the laboratory conditions is necessary. The models of the mentioned clinical situations provide new information on the pathophysiology of the disorders. Furthermore, they offer unique possibilities to test novel therapeutic approaches in the conditions very similar to the respective clinical syndromes.

* Corresponding author information (for footnote): mokra@jfmed.uniba.sk.

ABBREVIATIONS

A-a DO_2	Alveolar-arterial oxygen difference
ALI	Acute lung injury
ARDS	Acute respiratory distress syndrome
BAL	Bronchoalveolar lavage fluid
FiO_2	Fraction of inspired oxygen
HCl	Hydrochloric acid
IFNγ	Interferon gamma
IgM	Immunoglobulin M
IL	Interleukin
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
MAS	Meconium aspiration syndrome
MPO	Myeloperoxidase
NF-κB	Nuclear factor kappa-B
NO	Nitric oxide
$PaCO_2$	Arterial partial pressure of carbon dioxide
PaO2	Arterial partial pressure of oxygen
PEEP	Positive end-expiratory pressure
PIMs	Pulmonary intravascular macrophages
PMN	Polymorphonuclears
RDS	Respiratory distress syndrome
SpO_2	Oxygen saturation of hemoglobin
TNFα	Tumor necrosis factor alpha
VILI	Ventilator-induced lung injury

1. INTRODUCTION

An “acute lung injury” (ALI) and its more severe form “acute respiratory distress syndrome” (ARDS) represent life-threatening situations which can result from various reasons and occur in all age groups. These syndromes are characterized by a diffuse alveolar injury, a formation of lung edema, an inflammation, and a ventilation-perfusion mismatch. The mentioned changes finally lead to a decline in the lung compliance and in a profound hypoxemia (Ware and Matthay 2000).

An incidence of ARDS in adults varies between 30-80 per 100.000 population due to a regional genetic variability, a quality of health care as well as due to differences in the methodology and diagnostic criteria (Li et al. 2011, Villar et al. 2011, Standiford and Ward 2016). In newborns, the respiratory distress is also common, affecting up to 7% of all term newborns (Kumar and Bhat 1996) and is increasing with the prematurity. Although a significant progress in understanding the pathophysiology and the use of lung-protective ventilation techniques in adult patients has improved the situation, the incidence of ARDS is still relatively high (Standiford and Ward 2016). A similar situation with the respiratory distress can be observed in neonates, representing a serious therapeutic problem.

The use of appropriate animal models of ALI/ARDS brings new information on the pathophysiology of the respiratory distress and provides a unique possibility to evaluate novel therapeutic approaches. Furthermore, results from the modeling the clinical syndromes in the laboratory conditions can be later effectively transferred to the clinical settings.

2. ACUTE LUNG INJURY

2.1. Definitions of Acute Lung Injury

American-European Consensus Conference in 1994 (Bernard et al. 1994) postulated basic diagnostic criteria for clinical ARDS:

- an acute hypoxemia, defined as a ratio of arterial partial pressure of oxygen (PaO_2) and fraction of inspired oxygen (FiO_2); for ARDS: PaO_2/FiO_2 is <200 mmHg (26.7 kPa), for ALI: PaO_2/FiO_2 between 200 mmHg (26.7 kPa) and 300 mmHg (40 kPa)
- bilateral infiltrates on chest X-ray
- no increase in the pulmonary artery wedge pressure.

The Berlin Definition released in 2012 considered following three categories of ARDS according to the severity of hypoxemia: mild (PaO_2/FiO_2 200-300 mmHg), moderate (PaO_2/FiO_2 100-200 mmHg) and severe (PaO_2/FiO_2 less than 100 mmHg) form of ARDS (ARDS Definition Task Force 2012, Fioretto and de Carvalho 2013).

For experimental studies with artificially induced respiratory insufficiency and without other clinically relevant signs except of hypoxemia, the term "acute lung injury" is preferred.

2.2. Risk Factors and the Etiopathogenesis of ALI

ALI usually occurs from direct reasons, such as an aspiration of the gastric content, pneumonia, or inhalation of toxic gases as well as from indirect ones, which originate as a consequence of a serious systemic injury, e.g., in sepsis, severe trauma with shock, or pancreatitis (Table 1).

Table 1. Risk factors of ALI/ARDS in adult humans (Modified according to: Ware and Matthay 2000, Mortelliti and Manning 2002, Saharan et al. 2010)

Risk factors of ALI/ARDS	
Direct lung injury	Indirect lung injury
Often	**Often**
Pneumonia	Sepsis
Aspiration of gastric content	Severe trauma with shock
	Repetitive transfusions of blood products
Rare	**Rare**
Lung contusion	Acute pancreatitis
Fat embolism	Drug abuse
Near-drawning	Burns
Inhalational injury (smoke, gases)	Cardiopulmonary by-pass
Reperfusion edema after lung transplantation or lung embolectomy	Injury of head Disseminated intravascular coagulation (DIC)

In neonates, the respiratory distress can result from a delayed removal of the pulmonary fluid after delivery (transient tachypnea of the newborn), an immaturity of the lung tissue and from an insufficient production of the surfactant in the preterm neonates (respiratory distress syndrome, RDS), from intrauterine or perinatal aspirations of the meconium (the first stools of the neonate) into the lung of the term or post-term neonates (meconium aspiration syndrome, MAS). Other etiopathogenetic reasons include an infection

(pneumonia, sepsis), nonpulmonary causes (e.g., congenital heart diseases), or a persistent pulmonary hypertension etc. (Hermansen and Lorah 2007, Reuter et al. 2014). In children and adults, the most frequent causes of ALI are represented by a pneumonia, an aspiration of gastric content, sepsis or trauma with a shock (Marraro et al. 2014, Orwoll and Sapru 2016).

2.3. Pathophysiology and the Clinical Picture of ALI/ARDS in Adults and Children

An acute (exudative) phase of ALI/ARDS is characterized by a diffuse injury of alveolar epithelial and/or endothelial cells leading to the formation of a lung edema and a ventilation-perfusion mismatch and finally to the worsened lung compliance and hypoxemia (Dushianthan et al. 2011, Donahoe 2011, Pierrakos et al. 2012). Activated neutrophils, alveolar macrophages and fixed lung cells increase the production of cytokines (especially of IL-1β, -6, -8, TNFα), proteases, and reactive oxygen species. These further potentiate the lung tissue injury (Cross and Matthay 2011). The acute phase is within several days followed by a fibroprolipherative phase with a various degree of fibrosis, neovascularization, and healing (Dushianthan et al. 2011, Donahoe 2011, Pierrakos et al. 2012).

2.4. Pathophysiology and the Clinical Picture of ALI/ARDS in Neonates

A presentation of the respiratory distress in neonates is very similar to that observed in adults. The most common cause of the neonatal respiratory distress is a *transient tachypnea of the newborn* constituting about 40% of all cases (Kumar and Bhat 1996). This benign condition occurs immediately after or within two hours after the birth due to a delayed removal of the lung fluid after delivery. A tachypnea and diffuse parenchymal infiltrates, a "wet silhouette" around the heart, or an intralobar fluid accumulation on the chest radiography disappear within several hours up to two days (Kurl et al. 1997).

More severe form of the neonatal respiratory distress is a *respiratory distress syndrome of the newborn*, also called a hyaline membrane disease. This situation occurs in premature neonates due to the structural and functional lung immaturity, particularly in infants born before 28 weeks of gestation (Hermansen and Lorah 2007). Immature type II alveolar cells synthesize less

surfactant; this leads to an increased alveolar surface tension and decreased lung compliance. The resultant alveolar atelectasis causes a pulmonary vasoconstriction and a hypoperfusion. A proteinaceous fluid leaks through the injured alveolo-capillary membrane, clots and forms hyaline membranes. The persistent RDS leads to a bronchopulmonary dysplasia with typical chest radiography findings and chronic oxygen support (Koivisto et al. 2005). The clinical signs include hypoxia, cyanosis, grunting, retractions and other signs of the respiratory distress immediately after the birth. The chest radiography shows homogenous opaque infiltrates, air bronchograms and decreased lung volumes (Kurl et al. 1997).

Meconium-stained amniotic fluid occurs in 10-15% of all deliveries; however, *a meconium aspiration syndrome* (MAS) can develop just in some of those cases (Cleary and Wiswell 1998). This situation is typical for the term and post-term infants. The meconium can be evacuated from intestine into the amniotic fluid due to a hypoxia or a fetal distress *in utero*. From the same reasons the meconium-stained amniotic fluid can be aspirated *in utero* or during the labor. Despite the meconium is usually sterile, its components can cause obstruction of the airways and trigger a dysfunction of surfactant, pneumonitis, a lung edema and a pulmonary vasoconstriction immediately or several hours after delivery. The chest radiography shows patchy atelectases or consolidations (Cleary and Wiswell 1998, Swarnam et al. 2012).

Another reason for the respiratory insufficiency in neonates is a *bacterial pneumonia.* The infection is usually caused by group B *Streptococci*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and gram-negative enteric rods. The clinical picture may have various manifestations including signs of respiratory distress and a temperature instability. Contrary to previously mentioned situations, the pneumonia occurs hours to days after the delivery as the bacterial infection needs time to develop. The chest radiography can show bilateral infiltrates and pleural effusions (Hermansen and Lorah 2007).

3. MODELS OF ALI

Animal models provide an important bridge between the patients and the laboratory bench. Human studies bring fundamental descriptive information about the onset and the evolution of the pathophysiological and inflammatory changes in the lung. However, hypotheses about mechanisms of the injury have been difficult to test in humans but can be easily tested in the animals. The models of ALI reproduce the mechanisms and consequences of

ALI/ARDS in humans including the clinical, physiological, biological and pathological changes (Matute-Bello et al. 2008) (Table 2).

Nevertheless, while in humans the criteria for ALI are well defined, these cannot be directly translated to experimental animals. From this reason, an Official American Thoracic Society Workshop concluded that the main features of the experimental ALI include:

- a histological evidence of tissue injury,
- an alteration of the alveolar capillary membrane,
- a presence of an inflammatory response,
- an evidence of the physiological dysfunction (Matute-Bello et al. 2011, Table 3).

Table 2. Features of the acute lung injury in humans (modified according to Matute-Bello et al. 2008)

Clinical features	Acute onset Diffuse bilateral alveolar injury Acute exudative phase Repair with fibrosis
Physiological changes	Ventilation/perfusion mismatching Increase in the intrapulmonary shunt fraction Severe hypoxemia Decreased lung compliance Impaired clearance of alveolar fluid
Biological changes	Increased endothelial and epithelial permeability Increase in extravascular lung water Increased concentrations of proinflammatory cytokines in the lung Activation of proteases Abnormalities of the coagulation
Pathological changes	Neutrophil infiltration of alveoli Intra-alveolar coagulation and deposition of fibrin (formation of hyaline membranes) Abnormalities of the pulmonary surfactant Injury to the alveolar epithelium with denudation of the basement membrane

Table 3. Features of the acute lung injury in experimental animals (according to Matute-Bello et al. 2011)

Histological evidence of the tissue injury	Very relevant Accumulation of neutrophils in the alveolar or the interstitial space Formation of hyaline membranes Presence of proteinaceous debris in the alveolar space (such as fibrin strands) Thickening of the alveolar wall Enhanced injury as measured by a standardized histology score Somewhat relevant Evidence of the hemorrhage Areas of atelectases Gross macroscopic changes as a discoloration of the lungs
Alteration of the alveolar capillary membrane	Very relevant Increase in the extravascular lung water content Accumulation of an exogenous protein or tracer in the airspaces or the extra vascular compartment Increase in the total bronchoalveolar (BAL) protein concentration Increase in the concentration of high molecular weight proteins in BAL fluid (e.g., albumin, IgM) Increase in the microvascular filtration coefficient Somewhat relevant Increase in the lung wet/dry weight ratio Translocation of a protein from the airspaces into the plasma Increased lung lymph flow High lymph protein concentration
Inflammatory response	Very relevant Increase in the absolute number of neutrophils in BAL fluid Increase in the lung myeloperoxidase (MPO) activity or protein concentration Increase in the concentrations of proinflammatory cytokines in the lung tissue or BAL fluid Somewhat relevant Increase in the procoagulatory activity Increased expression of adhesion molecules Conversion of the neutrophilic alveolitis into a mononuclear alveolitis Increase in the levels of complement factors and matrix metalloproteinases
Evidence of the physiological dysfunction	Very relevant Hypoxemia (PaO_2 <60 mmHg or SpO_2 <90%) Increased alveolar-arterial oxygen difference [(Aa)DO_2] Somewhat relevant PaO_2/FiO_2 <200 Increase in the spontaneous minute ventilation Increase in the spontaneous respiratory rate

To determine if ALI has occured in animals, the Workshop recommended that at least three of the four "main features" of ALI were to be identified. To determine if any of the main features of ALI are present, the Workshop recommended using at least one of the "very relevant" measurements listed in the Table 3, and preferably, one or two additional separate measurements to confirm the results. However, not all of the measurements listed should be performed in every study (Matute-Bello et al. 2011, Table 3).

When modeling a human lung injury, the differences between the species should be considered. Matute-Bello et al. 2008 in their excellent article reviewed some unique characteristics of various animal species, i.e., the species differences in an innate immune response (differences in Toll-like receptors, in the mononuclear phagocyte system, in the production of nitric oxide, in chemokines and chemokine receptors) and the differences in animal sizes which might limit the value of the results obtained from the animal model.

According to the primary target, the animal models of ALI can be divided into three main groups:

- models with a primary dysfunction of the epithelium
- models with a primary dysfunction of the endothelium
- models with a dysfunction of both epithelium and endothelium (Matute-Bello et al. 2008).

3.1. Animal Models with the Lung Epithelium as a Primary Target

3.1.1. Model of the Surfactant Depletion Induced by a Saline Lavage

The model of the surfactant depletion is usually performed in anesthetized animals (rabbits, pigs, dogs, sheep etc.) after a short period of stabilization (e.g., 15 min) during which the animal is ventilated with an oxygen (FiO_2 1.0). The first measurement of respiratory parameters is done and blood samples are taken for a determination of the blood gases. The model of the surfactant depletion is induced by an instillation of a pre-warmed (37-39 °C) isotonic saline solution at the volume of 10-30 ml/kg through the endotracheal tube and its immediate suctioning. This procedure is repeated until a target degree of the hypoxemia, e.g., 200 mmHg (26.7 kPa) or 300 mmHg (40 kPa), is reached. The period of the restoration lasting several minutes should be left between the individual lavages. When the target hypoxemia is reached in the two

consecutive measurements 5 min and 15 min after the lavage, the model is considered to be prepared and the experiment continues with an administration of any pharmacological therapy or a use of any mode of the artificial ventilation (Rotta et al. 2001, Ronchi et al. 2011, Ronchi et al. 2012, Fioretto et al. 2012, Kosutova et al. 2016, Mokra et al. 2016). In several studies, a low amount of detergent, e.g., Tween 80 at a concentration of 0.2 or 0.5%, was added to further inactivate the pulmonary surfactant (Kobayashi et al. 1996, Musch et al. 2004).

The saline lung lavage removes a significant portion of the lung surfactant. This leads to an increase in the alveolar surface tension and an impairment of the alveolar host defence (Matute-Bello et al. 2008, Wang et al. 2008). The lung lavage facilitates the alveolar collapse and increases the likelihood of a mechanical injury to the alveolar walls during the repetitive opening and closure in the ventilation. These changes result in an immediate hypoxemia and increase of the A-a oxygen difference, as well as in an increased production of the lung edema, increased migration of polymorphonuclears (PMN) into the alveolar spaces and in elevated synthesis of pro-inflammatory cytokines (Rotta et al. 2001, Vangerow et al. 2001, Ronchi et al. 2011). In the histological investigation, the alveolar collapse changing with over distended regions and a peribronchial edema can be found. The alveolo-capillary membrane is thickened with a destruction and desquamation of epithelial and endothelial cells, with a necrosis of alveolar type I cells, and an injury to the basement membrane (Kuckelt et al. 1981, Imai et al. 2001).

A disadvantage of this model is the fact that except of neonates, the surfactant depletion rarely occurs in the absence of a severe alveolar damage. The surfactant depletion in adults can be a consequence of the primary injury to the lung epithelium and an exudation of the protein-rich edema fluid into the alveolar spaces. Contrary, in the lung lavage model the surfactant depletion is the primary cause of the injury (Matute-Bello et al. 2008). The technical disadvantage of this model is that the animals require administration of a general anaesthesia, an insertion of a tracheal cannula and an artificial ventilation. Nevertheless, this model is an optimal possibility for the testing of different ventilation strategies (Matute-Bello et al. 2008).

The model of the surfactant depletion by a repetitive saline lung lavage is particularly suitable for the simulation of an insufficient surfactant production and the lung injury observed in the premature neonates. Although in this model young-to-adult animals (particularly rabbits) are commonly used, this model is generally accepted for this purpose (Rotta et al. 2001, Ronchi et al.

2011, Ronchi et al. 2012, Fioretto et al. 2012, Kosutova et al. 2016, Mokra et al. 2016). The main advantage of this model is that the body weight, the diameter of the airways, and the lung compliance of rabbits are very close to those in the neonates. This allows a use of the artificial ventilation with comparable values of the ventilatory parameters as in neonates. Another advantage of this model is the possibility to obtain blood samples of a sufficient volume for measurement of blood gases and other parameters expressing the effectiveness of the used ventilation or other therapy repetitively during the experiment. On the other hand, in this model older animals than neonatal pups are usually used, what can limit the translational value of the results.

In our studies, the lung lavage with the saline (at a dose of 30 ml/kg, repeated 6-12 times) worsened the gas exchange, decreased the lung compliance and increased the intrapulmonary shunting. The lavage procedure triggered a neutrophil migration into the alveolar compartment and induced an inflammation and lung edema formation (Table 4).

Table 4. Changes in several inflammatory markers in the blood plasma and lung homogenate of healthy controls (Control) and saline-lavaged animals (LAV) 4 hours after the lavage (modified from Kosutova et al. 2016, Mokra et al. 2016 and Mokra et al. in press)

	Control	**LAV**
Wet-dry lung weight ratio	4.5 ± 0.1	6.0 ± 0.2***
Protein content in BAL (μg/ml)	308 ± 9	639.0 ± 16***
Total cell count in BAL (x 10^3/ml)	50.7 ± 10.0	677.5 ± 209.4**
Neutrophils in BAL (%)	1.8 ± 0.3	67.8 ± 5.1***
Monocytes-macrophages in BAL (%)	97.1 ± 0.8	27.0 ± 5.3***
Eosinophils in BAL (%)	1.1 ± 0.3	5.3 ± 1.3**
TNFα in lung (pg/ml)	293.1 ± 1.3	307.6 ± 0.4***
IL-1β in lung (pg/ml)	380.0 ± 46.7	688.1 ± 16.1***
IL-8 in lung (pg/ml)	438.5 ± 62.4	1144.7 ± 29.9***
IFNγ in lung (pg/ml)	688.4 ± 15.6	715.0 ± 4.2
TNFα in plasma (pg/ml)	286.8 ± 1.0	291.6 ± 1.0*
IL-1β in plasma (pg/ml)	15.2 ± 2.8	132.8 ± 32.0**
IL-8 in plasma (pg/ml)	19.4 ± 4.1	207.1 ± 43.6**

Abbreviations: BAL: bronchoalveolar lavage fluid, TNFα: tumor necrosis factor α, IL: interleukin, IFNγ: interferon gamma. For between-group differences: *P < 0.05, **P < 0.01, ***P < 0.001.

3.1.2. Model of the Lung Injury Induced by the Meconium

The meconium, i.e., the first feces of the neonate, is a viscous, dark green-black colored material which usually does not contain bacteria. Normally, the meconium is evacuated from the intestine within 2 days after the labor. However, due to an intrauterine stress (e.g., in fetal hypoxemia) or stimulation of *nervus vagus* the fetus increases the blood flow through the vitally important organs due to a vasoconstriction in the splanchnic circulation. Because of the ischemia of intestine, the meconium can pass into the amniotic fluid. In addition, the asphyxia stimulates respiratory movements of the fetus. The movements become irregular and while gasping the fetus can aspirate the amniotic fluid stained with the meconium. The meconium can be released into the amniotic fluid also in the post-term neonates, where the finding of the meconium-stained amniotic fluid without any symptoms of the respiratory distress can be considered as an indicator of the fetal maturation. The meconium-stained amniotic fluid can also be aspirated after the birth during first breaths (Cleary and Wiswell 1998, Klingner and Kruse 1999, Mokra and Mokry 2010).

In the acute phase after aspiration, particles of the inhaled meconium cause a mechanical obstruction of the airways leading to an increase in the airway resistance, atelectases of alveoli distally from the place of the occlusion, to the increased right-to-left pulmonary shunt and resulting hypoxemia, hypercapnia, and acidosis (Tran et al. 1980). With a passage of the meconium to peripheral regions of the lungs, a collapse of alveoli and small airways can occur due to the dysfunction of the surfactant (Moses et al. 1991, Lopez-Rodriguez et al. 2011), decrease in the dynamic lung compliance (Tyler et al. 1978), and a development of the non-infectious (or chemical) pneumonia with a neutrophil-mediated inflammation and cell death due to the apoptosis and necrosis (Zagariya et al. 2000, Soukka et al. 2002, Vidyasagar and Zagariya 2008, Jeng et al. 2008, Mollnes et al. 2008, Salvesen et al. 2010, Kopincova and Calkovska 2016), These changes are usually dependent on the amount and period of an exposure to the meconium.

The meconium consists of an amniotic fluid, desquamed epithelial cells, *dermis*, lanugo, *vernix caseosa*, mucus, bile acids and salts, bilirubin, cholesterol, free fatty acids, gastrointestinal enzymes including pancreatic phospholipase A_2 etc. (Co and Vidyasagar 1990). Many of these substances can injure the lung cells and surfactant directly. They can induce the inflammatory response in the lung, or produce pulmonary vasoconstriction (Davey et al. 1993, Holopainen et al. 1998, Kuo and Chen 1999, Mokra and Calkovska 2013). As a result of the mentioned changes, the permeability

through the injured alveolocapillary membrane increases and a lung edema is formed. The asphyxia, oxidative stress, inflammation, and hemodynamic changes associated with the meconium aspiration can be further responsible for changes in distant organs, e.g., in the brain hippocampus (Castellheim et al. 2005, Aaltonen et al. 2005a, Aaltonen et al. 2005b).

The models of meconium aspiration syndrome (MAS) are usually performed in anesthesized animals by an intratracheal instillation of a suspension of the meconium. The meconium is taken from diapers of healthy neonates, is pooled and lyophilized in very low temperatures in a lyophilizator. The lyophilized meconium powder is then suspended in a normal pre-heated (37 °C) saline to a required concentration. According to the animal species and design of experiment, various combinations of meconium concentrations and dose volumes can be used.

For instance, in adult rabbits an instillation of the meconium suspension with a concentration of 25 mg/ml and a dose of 4 ml/kg resulted in a serious worsening of the lung functions with a decrease in the lung compliance of 45% of the initial value, increase in the right-to-left pulmonary shunts and significant inflammatory changes (Mokra et al. 2007, Mikolka et al. 2013, Mokra et al. 2015, Li et al. 2015). The instillation of the same dose of the meconium caused around 50% decrease in the lung compliance in adult rats, which was consistent till the end of the experiment (Sun et al. 1994). In adult rabbits (Hummler et al. 2001, Robinson and Roberts, 2002) and in piglets (Holopainen et al. 1999, Nakamura et al. 2000, Shekerdemian et al. 2004), higher concentrations of the meconium (20 or 25% of original concentration) in the lower volume (e.g., 3 ml/kg) can be also used. The above mentioned concentration (25 mg/ml) representing about 10% of the original concentration of the meconium creates a model of MAS with an accentuated alveolar component of the syndrome and with the predominant surfactant dysfunction and inflammation. Contrary, an instillation of higher concentrations of the meconium generates the model with an accentuation of the airway obstruction (Davey et al. 1993). Before an administration, the meconium suspension can be filtered through a gauze or a blotting paper to separate large meconium particles, or it can be delivered non-filtered. The later possibility resembles better the situation of the meconium aspiration in the neonates, however, it is associated with an increase in $PaCO_2$ because of an airway obstruction by the meconium particles (Tyler et al. 1978, Sun et al. 1996, Sevecova-Mokra et al. 2004, Mokra et al. 2016).

Table 5. Changes in several inflammatory markers in the blood plasma and lung homogenate of healthy controls (Control) and meconium-instilled animals (MAS) 5 hours after the meconium instillation (modified from Mikolka et al. 2013, Mikolka et al. 2016 and Mokra et al. 2016)

	Control	**MAS**
Wet-dry lung weight ratio	4.7 ± 0.2	6.5 ± 0.2***
Protein content in BAL (μg/ml)	308 ± 9	524.9 ± 50.7**
Total cell count in BAL (x 10^3/ml)	221.7 ± 38.6	640.0 ± 141.0***
Neutrophils in BAL (%)	4.5 ± 3.1	69.0 ± 5.1***
Monocytes-macrophages in BAL (%)	94.0 ± 4.0	26.4 ± 4.5***
Eosinophils in BAL (%)	1.1 ± 0.9	2.9 ± 1.2**
TNFα in lung (pg/ml)	294.4 ± 1.3	309.1 ± 0.4***
IL-1β in lung (pg/ml)	85.6 ± 15.3	258.6 ± 3.8***
IL-8 in lung (pg/ml)	460.3 ± 55.4	1195.1 ± 39.2***
IFNγ in lung (pg/ml)	665.9 ± 25.9	681.7 ± 21.0
TNFα in plasma (pg/ml)	287.3 ± 0.6	313.3 ± 2.3***
IL-1β in plasma (pg/ml)	90.4 ± 19.5	126.8 ± 7.4*
IL-8 in plasma (pg/ml)	20.3 ± 2.8	1179.8 ± 162.3***

Abbreviations: BAL: bronchoalveolar lavage fluid, TNFα: tumor necrosis factor α, IL: interleukin, IFN: interferon. For between-group differences: *P < 0.05, **P < 0.01, ***P < 0.001.

Different animal species have been used for creation of MAS models. The use of neonatal animals immediately after the labor is of the biggest advantage, as these models simulate the aspiration of the meconium on the background of postnatal changes of the lungs from liquid-filled to air-filled organ (Sun et al. 1993). However, due to technical and ethical difficulties associated with this procedure, several days up to several weeks-old animals, e.g., piglets (Kuo and Chen 1999, Khan et al. 1999, Nakamura et al. 2000, Aaltonen et al. 2005a, Geiger et al. 2005), young rabbits (Krause et al. 1998, Vidyasagar and Zagariya 2008, Zagariya et al. 2010), rats (Cleary et al. 1997, Calkovska et al. 1999, Turhan et al. 2012), or rarely lambs (Rey-Santano et al. 2011) and mice (Khan et al. 2002) are used instead of animal pups immediately after the labor. On the other hand, utilization of several weeks-old or adult animals (particularly piglets and rabbits) can be advantageous. These models are to be considered, if different modes of the artificial ventilation are performed or treatments are delivered intratracheally, including the lung lavage with an exogenous surfactant etc. The benefit lies in the fact that piglets

and young or adult rabbits have similar size of the airways and similar body weight to those in the neonates (Sun et al. 1994, Sun et al. 1996, Chappell et al. 2001, Shekerdemian et al. 2004, Sevecova-Mokra et al. 2004, Mokra et al. 2007, Mikolka et al. 2013, Renesme et al. 2013, Mikusiakova et al. 2015, Mokra et al. 2016).

In our studies, an instillation of the meconium suspension (25 mg/ml, 4 ml/kg) significantly increased the number of cells (particularly neutrophils) in the bronchoalveolar lavage (BAL) fluid at the end of the experiment (5 hours after the meconium instillation) compared to healthy controls. In addition, increased concentrations of pro-inflammatory cytokines in the lung homogenate and in the blood plasma as well as an increased protein content in the BAL fluid and higher wet-dry lung weight ratio were detected (Table 5). Furthermore, the meconium instillation triggered a significant oxidative stress leading to a severe peroxidation of lipids and proteins in the lung tissue (Mokra et al. 2015, Mikolka et al. 2016).

3.1.3. Model of the Lung Injury Induced by a Hyperoxia

Whereas it is generally agreed that high concentrations of the oxygen can produce or exacerbate a lung injury in humans, the hyperoxia is used as a direct cause of an injury in animal studies. The injury to lungs originates from an action of the reactive oxygen species derived from the oxygen or from interactions with other species, e.g., nitric oxide (NO) (Matute-Bello et al. 2008). An abundant production of free radicals leads to an oxidation of proteins and peroxidation of membrane lipids and nucleic acids. These changes result in an apoptosis and necrosis of the lung cells (Barazzone et al. 1998), an increased translocation of NF-κB, and an elevated production of the pro-inflammatory cytokines (Shea et al. 1996).

To create a hyperoxia model, animals are housed in a sealed cage with higher PO_2. An exposure to the normobaric oxygen within 3-4 days leads to a generation of the exudative phase of ALI/ARDS with a damage to alveolar type I cells, necrosis of endothelial cells, interstitial and intraalveolar edema, an increased platelet adhesion and PMN accumulation (Crapo et al. 1980, Barry and Crapo 1985). In rats and mice, an exposure to the 100% oxygen for 40-50 hours resulted in ALI and longer exposure (60-70 hours) resulted in a death (Barry and Crapo 1985, Matute-Bello et al. 2008). A survival after the O_2 exposure is age-dependent whereas neonate animals were more tolerant to the hyperoxia. However, there were observed differences between the species. The tolerance was associated with an increase in the pulmonary concentrations

of antioxidant enzymes, such as manganese superoxide dismutase and glutathione reductase (Frank et al. 1977).

A disadvantage of this model is the fact that the exposure of humans to the 100% oxygen for up to 3 days does not result in a lung injury (Barber et al. 1970). Another limitation of this type of the model is the requirement for a specialized equipment to ensure a delivery of appropriate O_2 concentrations for longer time.

3.1.4. Model of the Lung Injury Induced by a Mechanical Ventilation

A mechanical ventilation, particularly the ventilation with excessive volumes or high pressures, can produce a lung injury and inflammation, which is called a ventilator-induced lung injury (VILI) (Dreyfuss and Saumon 1998). In the model of ALI induced by the mechanical ventilation, the overstretching of the alveolar walls results in endothelial and epithelial breaks and interstitial edema (Fu et al. 1992). Detachments of the endothelial cells from the basement membrane, death of the epithelial cells and denuding the basement membrane can be detectable within half an hour of the excessive ventilation (Dreyfuss et al. 1985). These changes are accompanied by an increased permeability and formation of hyaline membranes and increased migration and activation of the lung cells including neutrophils (Pugin et al. 1998, Vlahakis et al. 1999, Li et al. 2014). The extent of the lung injury depends on the used ventilatory volumes and pressures as well as on the level or absence of positive end-expiratory pressure (PEEP) (Petrucci and De Feo 2013, Santa Cruz et al. 2013). For instance, the large volume ventilation results in an alveolar hemorrhage, formation of hyaline membranes, neutrophilic infiltration, decline in the lung compliance and worsened gas exchange (Altemeier et al. 2004). Contrary, the ventilation with small volumes can reduce the inflammation and histopathological damages of the tissue (Ronchi et al. 2011).

There are several difficulties associated with the creation of this model. Animals should be in a general anesthesia, tracheotomized and ventilated with a mechanical ventilator, whereas ventilatory and cardiovascular parameters should be monitored. The other limitation is that the mechanical ventilation in humans is usually used as a treatment of ALI/ARDS which had originated from other reasons. Therefore, it is hardly to distinguish between consequences of the primary cause of ALI/ARDS and a iatrogenic VILI, while in the model of the lung injury induced by the mechanical ventilation the lung injury is primarily caused by the excessive mechanical ventilation.

3.1.5. Model of an Acid Aspiration

To simulate the aspiration of gastric contents in humans, an intratracheal instillation of hydrochloric acid (HCl) is generally used in animals. The intratracheal instillation of HCl with low pH (pH 1-2) induces an injury of the airway and alveolar epithelium and impairs an alveolar epithelial fluid transport (Modelska et al. 1999). The acid instillation also results in the injury to the capillary endothelium, probably through an action of neutrophils (Knight et al. 1992).

In most animal studies, 0.1 N HCl at a dose of 1-4 ml/kg is administered intratracheally. A lung injury induced by the instillation of HCl is a biphasic process (Reiss et al. 2012). The first phase reaches a peak within 1 hour after the HCl instillation and is characterized by an increase in the vascular permeability probably due to physiochemical reactions to the acid. The second peak is reached within 3-4 hours after the HCl instillation with a neutrophilic recruitment and an acute inflammatory response (Kennedy et al. 1989, Reiss et al. 2012). This is followed by an alveolar hemorrhage, intraalveolar and interstitial edema as well as by a decrease in the lung compliance, increased airway resistance and functional residual capacity, and hypoxemia (Folkesson et al. 1995, Rosenthal et al. 1998). The situation could be accompanied by an increased pulmonary vascular resistance and pulmonary artery pressure and elevated shunt fraction (Pawlik et al. 2005, Zarbock et al. 2006).

One of disadvantages of this model is that in addition to the HCl making low pH, the gastric content consists of other substances such as food particles, bacterial cell wall products, and cytokines, which participate in the aspiration-induced lung injury in humans (Raghavendran et al. 2005). To elicit a ALI model of the gastric content aspiration closer to the clinical situation, whole gastric fluid containing particles has been recently used in several studies (Davidson and Alluri 2013, Ayala et al. 2016).

3.1.6. Model of the Lung Injury Induced by an Intratracheal Bleomycin

The bleomycin model is generally considered as a model of the lung fibrosis, but it has some features of ALI, as well. Bleomycin forms a complex with the oxygen and metals leading to a generation of oxygen radicals, DNA breaks and a cell death (Burger et al. 1981).

Bleomycin can be delivered intravenously, intratracheally, intraperitoneally, or subcutaneously, most often in mice. While an intravenous administration targeting primarily the endothelium requires several weeks to exert required changes in the lung tissue, an intratracheal administration targeting primarily the epithelium can produce the lung fibrosis after a single

dose of bleomycin (Moore and Hogaboam 2008). In an acute phase after the intratracheal bleomycin administration, a patchy neutrophilic alveolitis and fibrosis can be detected. This is accompanied by increased neutrophils in the BAL fluid and elevated concentrations of pro-inflammatory cytokines (Shen et al. 1988). This model is technically relatively easy and reproducible, but due to its etiology has only a little relevance to clinics.

3.2. Animal Models with the Capillary Endothelium as a Primary Target

3.2.1. Model Induced by an Intravenous Administration of Lipopolysaccharide (LPS)

LPS is a glycolipid present in the outer membrane of Gram-negative bacteria. In the serum, LPS binds to a specific LPS binding protein (LBP) and forms a LPS:LBP complex. This complex activates a CD14/TLR4 receptor structure on many cells including monocytes and macrophages, and triggers a production of pro-inflammatory mediators (Wright et al. 1990, Tapping et al. 2000).

Early after an intravenous administration of LPS, the capillary endothelium exerts signs of apoptosis preceding other tissue damage (Fujita et al. 1998, Wang et al. 2007). Furthermore, it triggers a release of a wide range of mediators including cytokines, adhesion molecules and tissue factors (Brigham and Meyrick 1986, Bannerman and Goldblum 2003). The initial phase after the intravenous LPS administration is characterized by a leukopenia, decreased cardiac output and decreased arterial pressure, and an increased pressure in the pulmonary artery due to the increased resistance in the postcapillary veins (Kuida et al. 1958). The changes in leukocyte counts and hemodynamic parameters observed in the initial phase become stable within 4-6 hours. Within several hours after the intravenous delivery of the endotoxin, a hypoxemia and increased alveolar-arterial oxygen differences can be observed. Contrary to the intratracheal LPS delivery (see in chapter 3.3.1), the PMN infiltration into the lung tissue is relatively small (Matute-Bello et al. 2008). Interestingly, the endotoxin-induced ALI requires interaction with the liver. The direct effect of endotoxemia on the lung is just at the level of the pulmonary vasoconstriction and leukocyte sequestration. However, endotoxin causes an intense activation of the inflammation and oxidative injury in the lung, as both the liver and lungs are included in the blood-perfused circuit (Siore et al. 2005).

In humans, the intravenous administration of LPS at a dose of 2-4 ng/kg caused a systemic inflammation and priming of alveolar macrophages (Smith et al. 1994). The intravenous administration of 1-4 ng/kg of LPS in humans increased PMN, albumin and pro-inflammatory cytokines in the BAL fluid (O'Grady et al. 2001). In animals, the response to LPS can vary according to the presence or absence of the pulmonary intravascular macrophages (PIMs). Animal species with PIMs (sheep, cattle, pigs, cats, goats, horses etc.) can develop a pulmonary inflammation after very small doses of LPS (in μg/kg range), while species without PIMs (humans, dogs, rats, mice, rabbits etc.) require much higher doses (in mg/kg range) (Matute-Bello et al. 2008, Wang et al. 2008).

A big advantage of this model is its high reproducibility. LPS activates innate immune responses through the TLR4 pathways and has a low direct toxicity to cells *in vitro*. However, the intravenous LPS administration does not provide such severe injury to the endothelium and epithelium as observed in the human ARDS induced by living bacteria (Matute-Bello et al. 2008).

3.2.2. Model of an Oleic-Acid Lung Injury

The model of an oleic-acid lung injury serves as a model of the pulmonary lipid embolism in patients with a long bone trauma. This model is created by an intravenous administration of the oleic acid. It can be administered via a peripheral or central vein or directly into the right atrium or the pulmonary artery (Mattute-Bello 2008). The widely adopted dose of the oleic acid in the range of 0.06-0.15 ml/kg is mixed thoroughly with a normal saline and is injected slowly within 20-30 min. Alternatively, it can be administered continuously by an injector, or partionated into 3-4 equal aliquots (Wang et al. 2008). Because the oleic acid is insoluble in water, some researchers dissolve it in ethanol or emulsify it in the blood prior to the administration (Matute-Bello et al. 2008). Regardless of the route of administration, the effects of the oleic acid are detectable immediately, whereas ALI reaches maximum at 12 h and decreases towards 24 h (Derks and Jacobovitz-Derks 1977).

The exact mechanisms by which oleic acid induces the lung edema and an injury to the lung have not been completely elucidated yet. There are several presumed mechanisms which can contribute to this type of injury: an unsaturation of the oleic acid and its direct binding to biological membranes (Schuster 1994), a covalent binding to sodium channels and Na^+-K^+ ATPase in the epithelial cells and thereby impairing the sodium transport and edema formation (Vadász et al. 2005).

Early after the administration, the oleic acid causes severe vacuolization of the endothelial cells and necrosis (Hussain et al. 1998). The endothelial injury is followed by an epithelial injury with swelling and necrosis of type I alveolar cells. Within 30 min the oleic acid becomes detectable in the alveoli in the extracellular space and in the air spaces (Beilman 1995). The pulmonary microvascular permeability increases and a protein-rich fluid leaks into the interstitium and air spaces, whereas the increase in the alveolar permeability is proportional to the dose of administered oleic acid (Ehrhart and Hofman 1981). All mentioned changes lead to severe alterations in a gas exchange, hypoxemia, respiratory acidosis, and in increased alveolar-arterial oxygen difference within 90 min of the intravenous administration. This is caused by a severe ventilation/perfusion mismatching, decreased lung compliance, and elevated intrapulmonary shunt. Furthermore, hemodynamic changes such as myocardial depression, systemic hypotension, pulmonary hypertension, and elevated levels of circulating angiotensin converting enzyme can be detected early after the instillation of the oleic acid (Curtis and Peek 1994, Lamm et al. 1994).

The advantages of this model include early and rapid elicitation of ALI and a high reproducibility in different animals. One of the disadvantages of the model is the fact that the incidence of ALI/ARDS associated with long bone trauma or lipid injury is very rare.

3.3. Animal Models with Targeting of Both Epithelium and Endothelium

3.3.1. Models of the Lung Injury Due to a Sepsis

The sepsis in an animal model can be induced by an administration of living bacteria, by a creation of an endogenous infection, e.g., caecal ligation and puncture, or by an administration of bacterial products, e.g., endotoxin (see in a subchapter 3.2.1).

The models using living bacteria can differentiate according to the route of administration, the size of bacterial inoculum, the bacterial species, and the animal species (Matute-Bello et al. 2008). An intravenous administration of bolus of bacteria is followed by a hypotension and leukopenia, which can progress to a septic shock, intravascular coagulation and death. If the animal survives, an acute phase is followed by a hemodynamic stabilization with a microvascular injury and PMN lung sequestration, an increased pulmonary vascular permeability, an increase in the shunt fraction and pulmonary artery

pressure, and an intravascular thrombosis (Welty-Wolf et al. 2008). However, in this model the alveolar epithelium is relatively resistant to the injury (Wiener-Kronish et al. 1991). Thus, the experimental bacteriemia is usually not associated with the full histopathological picture of ARDS, including the epithelial damage and formation of hyaline membranes. Although the sepsis is a major risk factor for ARDS, the bacteriemia is either not present or less severe than observed in the animal models (Montgomery et al. 1985).

A model of the lung injury secondary to the peritonitis can be induced surgically by the ligating and perforating the caecum, or rarely, bacteria can be incorporated into a carrier vehicle such as a fibrin clot or a sponge placed into the peritoneum (Eichacker et al. 1994, Matute-Bello et al. 2001). After the perforation of the caecum, the features of the peritonitis develop within several days and the onset is less abrupt. A leukopenia and pulmonary hypertension develop within 1-2 days and the lung injury within 3 days. These changes are associated with a hypoxemia, neutrophilic inflammation, and interstitial and alveolar edema (Goya et al. 1992, Lomas-Neira et al. 2005). In this model, the lung injury similar to ALI/ARDS can be found. However, it is accompanied by less severe intra-alveolar inflammation and formation of hyaline membranes (Matute-Bello et al. 2008).

In the study carried out by our research team, an intraperitoneal administration of LPS at a dose 100 μg/kg of the body weight in adult rats evoked a monophasic thermic response (LPS-induced fever) and increased the minute ventilation due to changes in the breathing rate and the tidal volume. The LPS-instilled animals had higher levels of surfactant proteins SP-A, SP-B and SP-D in the lung tissue and higher SP-C and SP-D in the BAL fluid than the saline-instilled controls. The alterations of proteins related to the local immune mechanisms (SP-A, SP-D) were probably a part of the general inflammatory response to pyrogen, while the changes in proteins related to the surface activity (SP-B and SP-C) might reflect the effort of the body to stabilize the lungs in thermal challenge (Kolomaznik et al. 2014). In addition, the endotoxemia in rats was accompanied by changes in the heart rate variability, an index of cardiac autonomic control. We found a significant decrease in the spectral activity in high-frequency range at maximum body temperature and increased IL-6 in the heart tissue homogenates of the LPS-injected rats. These changes may indicate a decreased parasympathetic activity in the LPS-induced endotoxemia as a basic characteristic of the altered cardiac control during a response to the endotoxemia (Zila et al. 2015).

A local administration of living bacteria into the lungs results in a pneumonia and accorging to the size of the bacterial inoculum could exert

systemic manifestations of the sepsis (Fox-Dewhurst et al. 1997). The bacteria or LPS could be delivered to the lungs of animals by three different routes: an exposure to aerosol, an intratracheal administration, or an infection via the intranasal route. For the exposure to aerosol, a special equipment including a whole body exposure chamber and a nebulizer should be used. There are several advantages of this method: multiple animals can be infected simultaneously, bacteria reach symmetrically both lungs, and anesthesia is not required. However, other organs (e.g., eyes) are also exposed to microbes using this way of administration. Furhermore, some microbes sensitive to the high humidity might not survive when aerosolized. The intratracheal administration of the LPS or bacteria requires animals to be anesthesized and tracheotomized, or trachea should be punctured. On the other hand, the dose of the LPS or bacteria delivered into the lungs is precisely known. The third method of delivery is the infection via the intranasal route under the anesthesia. The animal receives small drops of bacterial suspension into each nostril in upright position and after the sniffing the material is transported towards the lower respiratory tract. There are several disadvantages of this method: necessity for anesthesia, and uncertainty of symmetrical delivery of the material in the lungs. However, this method is technically rather simple and time-saving (Knapp 2009).

As mentioned in the subchapter 3.2.1, the susceptibility to the circulating endotoxin is dependent on the presence of PIMs and is different among the species. Therefore, the dose of LPS required to induce a lung inflammation may vary from μg to mg/kg of the body weight. However, the role of PIMs in ALI induced by the intratracheal instillation of LPS has not been fully understood yet (Reiss et al. 2012). Endotoxins activate the innate immune response via their activation of TLR4 receptors (Beutler and Rietchel 2003). The lung injury induced by LPS develops within several days. During the first 2 days after the LPS instillation, the number of neutrophils in the lung and concentrations of cytokines increase (Håkansson et al. 2012). In this early phase also the vascular permeability elevates with maximum albumin levels and lymphocyte counts in the lung around day 4 (Nakajima et al. 2010), while levels of TNFα reach the peak value already within several hours after the LPS instillation and precede the migration of neutrophils (Faffe et al. 2000).

3.3.2. Models of an Ischemia/Reperfusion

An ischemia followed by a reperfusion either in the lungs or in any distant vascular bed can result in the lung injury. Such form of the lung injury can occur after a lung transplantation, where the reimplantation response is

characterized by a non-cardiogenic lung edema, inflammation, and hypoxia (Siegleman et al. 1973, Matute-Bello et al. 2008). ALI can also develop following a repair of aortic aneurysms etc. In an elicitation of the model of ischemia/reperfusion, the lungs are subjected to the ischemia by a clamping the pulmonary artery (preserving the bronchial circulation), or by a clamping the hilum (this stops all blood flow). The ischemia of the inflated lung for 2 hours followed by 2 hours of the reperfusion causes a structural damage of the alveolar endothelium and epithelium (Neely and Kelth 1995). An extent of the lung injury depends on several factors: an inflation state of the lung (deflated or inflated), an extent of the ischemic bed (pulmonary, bronchial circulation, venous return), a duration of the ischemia and reperfusion, an experimental preparation (*in vivo* or isolated perfused lung), and an animal species (Matute-Bello et al. 2008). Interestingly, the ischemia/reperfusion injury of one lung results in an inflammatory response and permeability changes not only in the occluded lung but also in the contralateral lung (Sakao et al. 2001).

3.4. Combination of the Models

Several groups of researchers combine two or more models to simulate a situation which is closer to the clinical ARDS. However, in that models it is rather difficult to determine the extent to which the lung injury is caused by one insult (e.g., the saline lung lavage) and the second insult (e.g., mechanical ventilation), or by both (Matute-Bello et al. 2008).

On the other hand, in several models of ALI the mechanical ventilation is required for an elicitation of the model (e.g., in the model of ALI induced by a repetitive saline lavage or by an instillation of meconium). A potentially deleterious effect of the artificial ventilation can be minimized, if animals are ventilated with small volumes and low ventilatory pressures. Contrary, if saline-lavaged animals are ventilated with high volumes and no positive end-expiratory pressure (PEEP), it results in the lung injury similar to ARDS with an increased protein permability, PMN infiltration and formation of hyaline membranes (Imai et al. 2001, Rotta et al. 2001, Matute-Bello et al. 2008). Similarly, an instillation of LPS into the lungs of surfactant-depleted animals can increase an intensity of the inflammatory response (Cochrane and Revak 1999). Any changes related to the mechanical ventilation can be different in normal and inflamed lungs. Nevertheless, they are additive to the changes induced by other factor (e.g., LPS) and the resulting injury can be dependent

on the type and extent of the lung inflammation (Altemeier et al. 2005, Matute-Bello et al. 2008).

CONCLUSION

Several types of animal models can be used to simulate the changes in the acutely injured lungs of patients including neonates. ALI/ARDS in humans has usually complex etiopathogenesis. The lung of the patient can be affected by a primary illness (e.g., sepsis) and/or can be affected also by therapeutic approaches (e.g., mechanical ventilation). Furthermore, the course of the syndrome is influenced by hereditary factors, susceptibility to the agents, concomitant diseases etc. Therefore, no single animal model can reproduce all characteristics of ALI/ARDS in humans, and most of the existing animal models are relevant for only limited aspects of ALI/ARDS in humans. Taking these limitations into account, the animal models are essential for an understanding the pathophysiology of ALI/ARDS and can provide an excellent opportunity to search for novel therapeutical strategies.

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The manuscript was reviewed by:

Jana Plevkova, PhD, Department of Pathological Physiology, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Martin, Slovakia

Juraj Mokry, PhD, Biomedical Center Martin and Department of Pharmacology, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Martin, Slovakia

REFERENCES

Aaltonen M, Soukka H, Halkola L, Jalonen J, Holopainen IE, Kääpä PO. Meconium aspiration induces oxidative injury in the hippocampus of newborn piglets. *Early Hum Dev* 2005a; 81(5): 439-447.

Aaltonen M, Soukka H, Halkola L, Kalimo H, Holopainen IE, Kääpä PO. Meconium aspiration induces neuronal injury in piglets. *Acta Paediatr* 2005b; 94(10): 1468-1475.

Altemeier WA, Matute-Bello G, Frevert CW, Kawata Y, Kajikawa O, Martin TR, Glenny RW. Mechanical ventilation with moderate tidal volumes synergistically increases lung cytokine response to systemic endotoxin. *Am J Physiol Lung Cell Mol Physiol* 2004; 287(3): L533-542.

Altemeier WA, Matute-Bello G, Gharib SA, Glenny RW, Martin TR, Liles WC. Modulation of lipopolysaccharide-induced gene transcription and promotion of lung injury by mechanical ventilation. *J Immunol* 2005; 175(5): 3369-3376.

ARDS Definition Task Force, Ranieri VM, Rubenfeld GD, Thompson BT, Ferguson ND, Caldwell E, Fan E, Camporota L, Slutsky AS. Acute respiratory distress syndrome: the Berlin Definition. *JAMA* 2012; 307(23): 2526-2533.

Ayala P, Meneses M, Olmos P, Montalva R, Droguett K, Ríos M, Borzone G. Acute lung injury induced by whole gastric fluid: hepatic acute phase response contributes to increase lung antiprotease protection. *Respir Res* 2016; 17(1): 71.

Bannerman DD, Goldblum SE. Mechanisms of bacterial lipopolysaccharide-induced endothelial apoptosis. *Am J Physiol Lung Cell Mol Physiol* 2003; 284(6): L899-914.

Barazzone C, Horowitz S, Donati YR, Rodriguez I, Piguet PF. Oxygen toxicity in mouse lung: pathways to cell death. *Am J Respir Cell Mol Biol* 1998; 19(4): 573-581.

Barber RE, Lee J, Hamilton WK. Oxygen toxicity in man. A prospective study in patients with irreversible brain damage. *N Engl J Med* 1970; 283(27): 1478-1484.

Barry BE, Crapo JD. Patterns of accumulation of platelets and neutrophils in rat lungs during exposure to 100% and 85% oxygen. *Am Rev Respir Dis* 1985; 132(3): 548-555.

Beilman G. Pathogenesis of oleic acid-induced lung injury in the rat: distribution of oleic acid during injury and early endothelial cell changes. *Lipids* 1995; 30: 817–823.

Bernard GR, Artigas A, Brigham KL, Carlet J, Falke K, Hudson L, Lamy M, LeGall JR, Morris A, Spragg R. Report of the American-European Consensus conference on acute respiratory distress syndrome: definitions, mechanisms, relevant outcomes, and clinical trial coordination. Consensus Committee. *J Crit Care* 1994; 9(1): 72-81.

Beutler B, Rietschel ET. Innate immune sensing and its roots: the story of endotoxin. *Nat Rev Immunol* 2003; 3(2): 169-176.

Brigham KL, Meyrick B. Endotoxin and lung injury. *Am Rev Respir Dis* 1986; 133(5): 913-927.

Burger RM, Peisach J, Horwitz SB. Activated bleomycin. A transient complex of drug, iron, and oxygen that degrades DNA. *J Biol Chem* 1981; 256(22): 11636-11644.

Calkovska A, Sun B, Curstedt T, Renheim G, Robertson B. Combined effects of high-frequency ventilation and surfactant treatment in experimental meconium aspiration syndrome. *Acta Anaesthesiol Scand* 1999; 43(2): 135-145.

Castellheim A, Lindenskov PH, Pharo A, Aamodt G, Saugstad OD, Mollnes TE. Meconium aspiration syndrome induces complement-associated systemic inflammatory response in newborn piglets. *Scand J Immunol* 2005; 61(3): 217-225.

Chappell SE, Wolfson MR, Shaffer TH. A comparison of surfactant delivery with conventional mechanical ventilation and partial liquid ventilation in meconium aspiration injury. *Respir Med* 2001; 95(7): 612-617.

Cleary GM, Antunes MJ, Ciesielka DA, Higgins ST, Spitzer AR, Chander A. Exudative lung injury is associated with decreased levels of surfactant proteins in a rat model of meconium aspiration. *Pediatrics* 1997; 100(6): 998-1003.

Cleary GM, Wiswell TE. Meconium-stained amniotic fluid and the meconium aspiration syndrome. An update. *Pediatr Clin North Am* 1998; 45: 511–529.

Co E, Vidyasagar D. Meconium aspiration syndrome. *Compr Ther* 1990; 16(10): 34-39.

Cochrane CG, Revak SD. Surfactant lavage treatment in a model of respiratory distress syndrome. *Chest* 1999; 116(1 Suppl): 85S-86S.

Crapo JD, Barry BE, Foscue HA, Shelburne J. Structural and biochemical changes in rat lungs occurring during exposures to lethal and adaptive doses of oxygen. *Am Rev Respir Dis* 1980; 122(1): 123-143.

Cross LJ, Matthay MA. Biomarkers in acute lung injury: insights into the pathogenesis of acute lung injury. *Crit Care Clin* 2011; 27(2): 355-377.

Curtis SE, Peek JT. Effects of progressive intratracheal administration of perflubron during conventional gas ventilation in anesthetized dogs with oleic acid injury. *Adv Exp Med Biol* 1994; 345: 51–58.

Davey AM, Becker JD, Davis JM. Meconium aspiration syndrome: physiological and inflammatory changes in a newborn piglet model. *Pediatr Pulmonol* 1993; 16(2): 101-108.

Davidson BA, Alluri R. Gastric Aspiration Models. *Bio Protoc* 2013; 3(22). pii: e968.

Derks CM, Jacobovitz-Derks D. Embolic pneumopathy induced by oleic acid A systematic morphologic study. *Am J Pathol* 1977; 87: 143-158.

Donahoe M. Acute respiratory distress syndrome: A clinical review. *Pulm Circ* 2011; 1: 192-211.

Dreyfuss D, Basset G, Soler P, Saumon G. Intermittent positive-pressure hyperventilation with high inflation pressures produces pulmonary microvascular injury in rats. *Am Rev Respir Dis* 1985; 132(4): 880-884.

Dreyfuss D, Saumon G. Ventilator-induced lung injury: lessons from experimental studies. *Am J Respir Crit Care Med* 1998; 157(1): 294-323.

Dushianthan A, Grocott MP, Postle AD, Cusack R. Acute respiratory distress syndrome and acute lung injury. *Postgrad Med J* 2011; 87: 612-622.

Eichacker PQ, Waisman Y, Natanson C, Farese A, Hoffman WD, Banks SM, MacVittie TJ. Cardiopulmonary effects of granulocyte colony-stimulating factor in a canine model of bacterial sepsis. *J Appl Physiol* (1985) 1994; 77(5): 2366-2373.

Ehrhart IC, Hofman WF. Oleic acid dose-related edema in isolated canine lung perfused at constant pressure. *J Appl Physiol* 1981; 50: 1115-1120.

Faffe DS, Seidl VR, Chagas PS, Gonçalves de Moraes VL, Capelozzi VL, Rocco PR, Zin WA. Respiratory effects of lipopolysaccharide-induced inflammatory lung injury in mice. *Eur Respir J* 2000; 15(1): 85-91.

Fioretto JR, Campos FJ, Ronchi CF, Ferreira AL, Kurokawa CS, Carpi MF, Moraes MA, Bonatto RC, Defaveri J, Yeum KJ. Effects of inhaled nitric oxide on oxidative stress and histopathological and inflammatory lung injury in a saline-lavaged rabbit model of acute lung injury. *Respir Care* 2012; 57(2): 273-281.

Fioretto JR, de Carvalho WB. ARDS definitions in children: one step forward. *J Pediatr* (Rio J) 2014; 90(2): 211-212.

Folkesson HG, Matthay MA, Hébert CA, Broaddus VC. Acid aspiration-induced lung injury in rabbits is mediated by interleukin-8-dependent mechanisms. *J Clin Invest* 1995; 96(1): 107-116.

Fox-Dewhurst R, Alberts MK, Kajikawa O, Caldwell E, Johnson MC 2nd, Skerrett SJ, Goodman RB, Ruzinski JT, Wong VA, Chi EY, Martin TR. Pulmonary and systemic inflammatory responses in rabbits with gram-negative pneumonia. *Am J Respir Crit Care Med* 1997; 155(6): 2030-2040.

Frank L, Bucher JR, Roberts RJ. Oxygen toxicity in neonatal and adult animals of various species. *J Appl Physiol Respir Environ Exerc Physiol* 1978; 45(5): 699-704.

Fu Z, Costello ML, Tsukimoto K, Prediletto R, Elliott AR, Mathieu-Costello O, West JB. High lung volume increases stress failure in pulmonary capillaries. *J Appl Physiol* (1985) 1992; 73(1): 123-133.

Fujita M, Kuwano K, Kunitake R, Hagimoto N, Miyazaki H, Kaneko Y, Kawasaki M, Maeyama T, Hara N. Endothelial cell apoptosis in lipopolysaccharide-induced lung injury in mice. *Int Arch Allergy Immunol* 1998; 117(3): 202-208.

Geiger R, Pajk W, Neu N, Maier S, Kleinsasser A, Fratz S, Navarro-Psiha S, Fischer V, Treml B, Loeckinger A. Tezosentan decreases pulmonary artery pressure and improves survival rate in an animal model of meconium aspiration. *Pediatr Res* 2006; 59(1): 147-150.

Goya T, Abe M, Shimura H, Torisu M. Characteristics of alveolar macrophages in experimental septic lung. *J Leukoc Biol* 1992; 52(2): 236-243.

Håkansson HF, Smailagic A, Brunmark C, Miller-Larsson A, Lal H. Altered lung function relates to inflammation in an acute LPS mouse model. *Pulm Pharmacol Ther* 2012; 25(5): 399-406.

Hermansen CL, Lorah KN. Respiratory distress in the newborn. *Am Fam Physician* 2007; 76(7): 987-994.

Holopainen R, Aho H, Laine J, Peuravuori H, Soukka H, Kääpä P. Human meconium has high phospholipase A2 activity and induces cellular injury and apoptosis in piglet lungs. *Pediatr Res* 1999; 46(5): 626-632.

Holopainen R, Soukka H, Halkola L, Kääpä P. Meconium aspiration induces a concentration-dependent pulmonary hypertensive response in newborn piglets. *Pediatr Pulmonol* 1998; 25(2): 107-113.

Hummler HD, Thome U, Schulze A, Schnabel R, Pohlandt F, Franz AR. Spontaneous breathing during partial liquid ventilation in animals with meconium aspiration. *Pediatr Res* 2001; 49(4): 572-580.

Hussain N, Wu F, Zhu L, Thrall RS, Kresch MJ. Neutrophil apoptosis during the development and resolution of oleic acid-induced acute lung injury in the rat. *Am J Respir Cell Mol Biol* 1998; 19: 867–874.

Imai Y, Nakagawa S, Ito Y, Kawano T, Slutsky AS, Miyasaka K. Comparison of lung protection strategies using conventional and high-frequency oscillatory ventilation. *J Appl Physiol* (1985) 2001; 91(4): 1836-1844.

Jeng MJ, Soong WJ, Lee YS, Tsao PC, Yang CF, Chiu SY, Tang RB. Meconium exposure dependent cell death and apoptosis in human alveolar epithelial cells. *Pediatr Pulmonol* 2010; 45(8): 816-823.

Kennedy TP, Johnson KJ, Kunkel RG, Ward PA, Knight PR, Finch JS. Acute acid aspiration lung injury in the rat: biphasic pathogenesis. *Anesth Analg* 1989; 69(1): 87-92.

Khan AM, Elidemir O, Epstein CE, Lally KP, Xue H, Blackburn M, Larsen GL, Colasurdo GN. Meconium aspiration produces airway hyperresponsiveness and eosinophilic inflammation in a murine model. *Am J Physiol Lung Cell Mol Physiol* 2002; 283(4): L785-790.

Khan AM, Shabarek FM, Kutchback JW, Lally KP. Effects of dexamethasone on meconium aspiration syndrome in newborn piglets. *Pediatr Res* 1999; 46(2): 179-183.

Klingner MC, Kruse J. Meconium aspiration syndrome: pathophysiology and prevention. *J Am Board Fam Pract* 1999; 12(6): 450-466.

Knapp S. LPS and bacterial lung inflammation models. Drug Discovery Today: *Disease Models* 2009; 6(4): 113-118.

Knight PR, Druskovich G, Tait AR, Johnson KJ. The role of neutrophils, oxidants, and proteases in the pathogenesis of acid pulmonary injury. *Anesthesiology* 1992; 77(4): 772-778.

Kobayashi H, Tanaka N, Winkler M, Zapol WM. Combined effects of NO inhalation and intravenous PGF2 alpha on pulmonary circulation and gas exchange in an ovine ARDS model. *Intensive Care Med* 1996; 22(7): 656-663.

Koivisto M, Marttila R, Saarela T, Pokela ML, Valkama AM, Hallman M. Wheezing illness and re-hospitalization in the first two years of life after neonatal respiratory distress syndrome. *J Pediatr* 2005; 147: 486–492.

Kolomaznik M, Zila I, Kopincova J, Mokra D, Calkovska A. Changes in lung surfactant proteins in rats with lipopolysaccharide-induced fever. *Physiol Res* 2014; 63 Suppl 4: S619-628.

Kopincova J, Calkovska A. Meconium-induced inflammation and surfactant inactivation: specifics of molecular mechanisms. *Pediatr Res* 2016; 79(4): 514-521.

Kosutova P, Mikolka P, Kolomaznik M, Balentova S, Calkovska A, Mokra D. Effects of S-Nitroso-N-Acetyl-Penicillamine (SNAP) on Inflammation,

Lung Tissue Apoptosis and iNOS Activity in a Rabbit Model of Acute Lung Injury. *Adv Exp Med Biol* 2016; 935: 13-23.

Krause MF, Lienhart HG, Haberstroh J, Hoehn T, Schulte-Mönting J, Leititis JU. Effect of inhaled nitric oxide on intrapulmonary right-to-left-shunting in two rabbit models of saline lavage induced surfactant deficiency and meconium instillation. *Eur J Pediatr* 1998; 157(5): 410-415.

Kuckelt W, Dauberschmidt R, Bender V, Hieronymi U, Mrochen H, Winsel K, Meyer M. Experimental investigations in adult respiratory distress syndrome (ARDS). Repeated pulmonary lavage in LEWE-mini-pigs. I. Pulmonary mechanics, gas exchange, and pulmonary hemodynamics. *Exp Pathol* 1981; 20(2): 88-104.

Kuida H, Hinshaw LB, Gilbert RP, Visscher MB. Effect of gram-negative endotoxin on pulmonary circulation. *Am J Physiol* 1958; 192(2): 335-344.

Kumar A, Bhat BV. Epidemiology of respiratory distress of newborns. *Indian J Pediatr* 1996; 63: 93–98.

Kuo C, Chen J. Effect of meconium aspiration on plasma endothelin-1 level and pulmonary hemodynamics in a piglet model. *Biol Neonate* 1999; 76(4): 228-234.

Kurl S, Heinonen KM, Kiekara O. The first chest radiograph in neonates exhibiting respiratory distress at birth. *Clin Pediatr* (Phila) 1997: 285–289.

Lamm WJ, Graham MM, Albert RK. Mechanism by which the prone position improves oxygenation in acute lung injury. *Am J Respir Crit Care Med* 1994; 150: 184-193.

Li LF, Lai YT, Chang CH, Lin MC, Liu YY, Kao KC, Tsai YH. Neutrophil elastase inhibitor reduces ventilation-induced lung injury via nuclear factor-κB and NF-κB repressing factor in *mice*. *Exp Biol Med* (Maywood) 2014; 239(8): 1045-1057.

Li G, Malinchoc M, Cartin-Ceba R, Venkata CV, Kor DJ, Peters SG, Hubmayr RD, Gajic O. Eight-year trend of acute respiratory distress syndrome: a population-based study in Olmsted County, Minnesota. *Am J Respir Crit Care Med* 2011; 183(1): 59-66.

Li AM, Zhang LN, Li WZ. Amelioration of meconium-induced acute lung injury by parecoxib in a rabbit model. *Int J Clin Exp Med* 2015; 8(5): 6804-6812.

Lomas-Neira J, Chung CS, Perl M, Gregory S, Biffl W, Ayala A. Role of alveolar macrophage and migrating neutrophils in hemorrhage-induced priming for ALI subsequent to septic challenge. *Am J Physiol Lung Cell Mol Physiol* 2006; 290(1): L51-58.

Lopez-Rodriguez E, Echaide M, Cruz A, Taeusch HW, Perez-Gil J. Meconium impairs pulmonary surfactant by a combined action of cholesterol and bile acids. *Biophys J* 2011; 100(3): 646-655.

Marraro GA, Chen C, Piga MA, Qian Y, Spada C, Genovese U. Acute respiratory distress syndrome in the pediatric age: an update on advanced treatment. *Chin J Contemp Pediatr* 2014; 16(5): 437-447.

Matute-Bello G, Downey G, Moore BB, Groshong SD, Matthay MA, Slutsky AS, Kuebler WM; Acute Lung Injury in Animals Study Group. An official American Thoracic Society workshop report: features and measurements of experimental acute lung injury in animals. *Am J Respir Cell Mol Biol* 2011; 44(5): 725-738.

Matute-Bello G, Frevert CW, Kajikawa O, Skerrett SJ, Goodman RB, Park DR, Martin TR. Septic shock and acute lung injury in rabbits with peritonitis: failure of the neutrophil response to localized infection. *Am J Respir Crit Care Med* 2001; 163(1): 234-243.

Matute-Bello G, Frevert CW, Martin TR. Animal models of acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2008; 295(3): L379-399.

Mikolka P, Mokra D, Kopincova J, Tomcikova-Mikusiakova L, Calkovska A. Budesonide added to modified porcine surfactant Curosurf may additionally improve the lung functions in meconium aspiration syndrome. *Physiol Res* 2013; 62 Suppl 1: S191-200.

Mikolka P, Kopincova J, Tomcikova Mikusiakova L, Kosutova P, Antosova M, Calkovska A, Mokra D. Effects of surfactant/budesonide therapy on oxidative modifications in the lung in experimental meconium-induced lung injury. *J Physiol Pharmacol* 2016; 67(1): 57-65.

Mikusiakova LT, Pistekova H, Kosutova P, Mikolka P, Calkovska A, Mokra D. Effects on Lung Function of Small-Volume Conventional Ventilation and High-Frequency Oscillatory Ventilation in a Model of Meconium Aspiration Syndrome. *Adv Exp Med Biol* 2015; 866: 51-59.

Modelska K, Pittet JF, Folkesson HG, Courtney Broaddus V, Matthay MA. Acid-induced lung injury. Protective effect of anti-interleukin-8 pretreatment on alveolar epithelial barrier function in rabbits. *Am J Respir Crit Care Med* 1999; 160(5 Pt 1): 1450-1456.

Mokra D, Calkovska A. How to overcome surfactant dysfunction in meconium aspiration syndrome? *Respir Physiol Neurobiol* 2013; 187(1): 58-63.

Mokra D, Drgova A, Mokry J, Antosova M, Durdik P, Calkovska A. N-acetylcysteine effectively diminished meconium-induced oxidative stress in adult rabbits. *J Physiol Pharmacol* 2015; 66(1): 101-110.

Mokra D, Kosutova P, Balentova S, Adamkov M, Mikolka P, Mokry J, Antosova M, Calkovska A. Effects of budesonide on the lung functions, inflammation and apoptosis in a saline-lavage model of acute lung injury. *J Physiol Pharmacol* – in press.

Mokra D, Mikusiakova LT, Mikolka P, Kosutova P, Jurcek M, Kolomaznik M, Calkovska A. High-Frequency Jet Ventilation against Small-Volume Conventional Mechanical Ventilation in the Rabbit Models of Neonatal Acute Lung Injury. *Adv Exp Med Biol* 2016; 912: 83-93.

Mokra D, Mokry J. Meconium aspiration syndrome: from pathomechanisms to treatment. New York: Nova Biomedical Books, 2010.

Mokra D, Mokry J, Drgova A, Petraskova M, Bulikova J, Calkovska A. Intratracheally administered corticosteroids improve lung function in meconium-instilled rabbits. *J Physiol Pharmacol* 2007; 58 Suppl 5(Pt 1): 389-398.

Mollnes TE, Castellheim A, Lindenskov PH, Salvesen B, Saugstad OD. The role of complement in meconium aspiration syndrome. *J Perinatol* 2008; 28 Suppl 3: S116-119.

Montgomery AB, Stager MA, Carrico CJ, Hudson LD. Causes of mortality in patients with the adult respiratory distress syndrome. *Am Rev Respir Dis* 1985; 132(3): 485-489.

Moore BB, Hogaboam CM. Murine models of pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2008; 294(2): L152-160.

Mortelliti MP, Manning HL. Acute respiratory distress syndrome. *Am Fam Physician* 2002; 65(9): 1823-1830.

Moses D, Holm BA, Spitale P, Liu MY, Enhorning G. Inhibition of pulmonary surfactant function by meconium. *Am J Obstet Gynecol* 1991; 164(2): 477-481.

Musch G, Harris RS, Vidal Melo MF, O'Neill KR, Layfield JD, Winkler T, Venegas JG. Mechanism by which a sustained inflation can worsen oxygenation in acute lung injury. *Anesthesiology* 2004; 100(2): 323-330.

Nakajima T, Suarez CJ, Lin KW, Jen KY, Schnitzer JE, Makani SS, Parker N, Perkins DL, Finn PW. T cell pathways involving CTLA4 contribute to a model of acute lung injury. *J Immunol* 2010; 184(10): 5835-5841.

Nakamura T, Matsuzawa S, Sugiura M, Tamura M. A randomised control study of partial liquid ventilation after airway lavage with exogenous surfactant in a meconium aspiration syndrome animal model. *Arch Dis Child Fetal Neonatal Ed* 2000; 82(2): F160-162.

Neely CF, Keith IM. A1 adenosine receptor antagonists block ischemia-reperfusion injury of the lung. *Am J Physiol* 1995; 268(6 Pt 1): L1036-1046.

O'Grady NP, Preas HL, Pugin J, Fiuza C, Tropea M, Reda D, Banks SM, Suffredini AF. Local inflammatory responses following bronchial endotoxin instillation in humans. *Am J Respir Crit Care Med* 2001; 163(7): 1591-1598.

Orwoll BE, Sapru A. Biomarkers in Pediatric ARDS: Future Directions. *Front Pediatr* 2016; 4: 55.

Pawlik MT, Schreyer AG, Ittner KP, Selig C, Gruber M, Feuerbach S, Taeger K. Early treatment with pentoxifylline reduces lung injury induced by acid aspiration in rats. *Chest* 2005; 127(2): 613-621.

Petrucci N, De Feo C. Lung protective ventilation strategy for the acute respiratory distress syndrome. *Cochrane Database Syst Rev* 2013; (2): CD003844.

Pierrakos C, Karanikolas M, Scolletta S, Karamouzos V, Velissaris D. Acute respiratory distress syndrome: pathophysiology and therapeutic options. *J Clin Med Res* 2012; 4: 7-16.

Pugin J, Dunn I, Jolliet P, Tassaux D, Magnenat JL, Nicod LP, Chevrolet JC. Activation of human macrophages by mechanical ventilation in vitro. *Am J Physiol* 1998; 275(6 Pt 1): L1040-1050.

Raghavendran K, Davidson BA, Mullan BA, Hutson AD, Russo TA, Manderscheid PA, Woytash JA, Holm BA, Notter RH, Knight PR. Acid and particulate-induced aspiration lung injury in mice: importance of MCP-1. *Am J Physiol Lung Cell Mol Physiol* 2005; 289(1): L134-143.

Reiss LK, Uhlig U, Uhlig S. Models and mechanisms of acute lung injury caused by direct insults. *Eur J Cell Biol* 2012; 91(6-7): 590-601.

Renesme L, Elleau C, Nolent P, Fayon M, Marthan R, Dumas De La Roque E. Effect of high-frequency oscillation and percussion versus conventional ventilation in a piglet model of meconium aspiration. *Pediatr Pulmonol* 2013; 48(3): 257-264.

Reuter S, Moser C, Baack M. Respiratory distress in the newborn. *Pediatr Rev* 2014; 35(10): 417-428.

Rey-Santano C, Alvarez-Diaz FJ, Mielgo V, Murgia X, Lafuente H, Ruiz-Del-Yerro E, Valls-I-Soler A, Gastiasoro E. Bronchoalveolar lavage versus bolus administration of lucinactant, a synthetic surfactant in meconium aspiration in newborn lambs. *Pediatr Pulmonol* 2011; 46(10): 991-999.

Robinson TW, Roberts AM. Effects of exogenous surfactant on gas exchange and compliance in rabbits after meconium aspiration. *Pediatr Pulmonol* 2002; 33(2): 117-123.

Ronchi CF, dos Anjos Ferreira AL, Campos FJ, Kurokawa CS, Carpi MF, de Moraes MA, Bonatto RC, Defaveri J, Yeum KJ, Fioretto JR. High-frequency oscillatory ventilation attenuates oxidative lung injury in a rabbit model of acute lung injury. *Exp Biol Med* (Maywood) 2011; 236(10): 1188-1196.

Ronchi CF, Fioretto JR, Ferreira AL, Berchieri-Ronchi CB, Correa CR, Kurokawa CS, Carpi MF, Moraes MA, Yeum KJ. Biomarkers for oxidative stress in acute lung injury induced in rabbits submitted to different strategies of mechanical ventilation. *J Appl Physiol* (1985) 2012; 112(7): 1184-1190.

Rosenthal C, Caronia C, Quinn C, Lugo N, Sagy M. A comparison among animal models of acute lung injury. *Crit Care Med* 1998; 26(5): 912-916.

Rotta AT, Gunnarsson B, Fuhrman BP, Hernan LJ, Steinhorn DM. Comparison of lung protective ventilation strategies in a rabbit model of acute lung injury. *Crit Care Med* 2001; 29(11): 2176-2184.

Saharan S, Lodha R, Kabra SK. Management of acute lung injury/ARDS. *Indian J Pediatr* 2010; 77(11): 1296-1302.

Sakao Y, Kajikawa O, Martin TR, Nakahara Y, Hadden WA 3rd, Harmon CL, Miller EJ. Association of IL-8 and MCP-1 with the development of reexpansion pulmonary edema in rabbits. *Ann Thorac Surg* 2001; 71(6): 1825-1832.

Salvesen B, Stenvik J, Rossetti C, Saugstad OD, Espevik T, Mollnes TE. Meconium-induced release of cytokines is mediated by the TRL4/MD-2 complex in a CD14-dependent manner. *Mol Immunol* 2010; 47(6): 1226-1234.

Santa Cruz R, Rojas JI, Nervi R, Heredia R, Ciapponi A. High versus low positive end-expiratory pressure (PEEP) levels for mechanically ventilated adult patients with acute lung injury and acute respiratory distress syndrome. *Cochrane Database Syst Rev* 2013; (6): CD009098.

Schuster DP. ARDS: clinical lessons from the oleic acid model of acute lung injury. *Am J Respir Crit Care Med* 1994; 149(1): 245-260.

Sevecova-Mokra D, Calkovska A, Drgova A, Javorka M, Javorka K. Treatment of experimental meconium aspiration syndrome with surfactant lung lavage and conventional vs. asymmetric high-frequency jet ventilation. *Pediatr Pulmonol* 2004; 38(4): 285-291.

Shea LM, Beehler C, Schwartz M, Shenkar R, Tuder R, Abraham E. Hyperoxia activates NF-kappaB and increases TNF-alpha and IFN-gamma gene expression in mouse pulmonary lymphocytes. *J Immunol* 1996; 157(9): 3902-3908.

Shekerdemian LS, Ravn HB, Penny DJ. Interaction between inhaled nitric oxide and intravenous sildenafil in a porcine model of meconium aspiration syndrome. *Pediatr Res* 2004; 55(3): 413-418.

Shen AS, Haslett C, Feldsien DC, Henson PM, Cherniack RM. The intensity of chronic lung inflammation and fibrosis after bleomycin is directly related to the severity of acute injury. *Am Rev Respir Dis* 1988; 137(3): 564-571.

Siegleman SS, Sinha SB, Veith FJ. Pulmonary reimplantation response. *Ann Surg* 1973; 177(1): 30-36.

Siore AM, Parker RE, Stecenko AA, Cuppels C, McKean M, Christman BW, Cruz-Gervis R, Brigham KL. Endotoxin-induced acute lung injury requires interaction with the liver. *Am J Physiol Lung Cell Mol Physiol* 2005; 289(5): L769-776.

Smith PD, Suffredini AF, Allen JB, Wahl LM, Parrillo JE, Wahl SM. Endotoxin administration to humans primes alveolar macrophages for increased production of inflammatory mediators. *J Clin Immunol* 1994; 14(2): 141-148.

Soukka HR, Ahotupa M, Ruutu M, Kääpä PO. Meconium stimulates neutrophil oxidative burst. *Am J Perinatol* 2002; 19(5): 279-284.

Standiford TJ, Ward PA. Therapeutic targeting of acute lung injury and acute respiratory distress syndrome. *Transl Res* 2016; 167(1): 183-191.

Sun B, Curstedt T, Robertson B. Surfactant inhibition in experimental meconium aspiration. *Acta Paediatr* 1993; 82(2): 182-189.

Sun B, Curstedt T, Robertson B. Exogenous surfactant improves ventilation efficiency and alveolar expansion in rats with meconium aspiration. *Am J Respir Crit Care Med* 1996; 154(3 Pt 1): 764-770.

Sun B, Herting E, Curstedt T, Robertson B. Exogenous surfactant improves lung compliance and oxygenation in adult rats with meconium aspiration. *J Appl Physiol* (1985) 1994; 77(4): 1961-1971.

Swarnam K, Soraisham AS, Sivanandan S. Advances in the management of meconium aspiration syndrome. *Int J Pediatr* 2012; 2012: 359571.

Tapping RI, Akashi S, Miyake K, Godowski PJ, Tobias PS. Toll-like receptor 4, but not toll-like receptor 2, is a signaling receptor for Escherichia and Salmonella lipopolysaccharides. *J Immunol* 2000; 165(10): 5780-5787.

Tran N, Lowe C, Sivieri EM, Shaffer TH. Sequential effects of acute meconium obstruction on pulmonary function. *Pediatr Res* 1980; 14(1): 34-38.

Turhan AH, Atıcı A, Muşlu N, Polat A, Helvacı İ. The effects of pentoxifylline on lung inflammation in a rat model of meconium aspiration syndrome. *Exp Lung Res* 2012; 38(5): 250-255.

Tyler DC, Murphy J, Cheney FW. Mechanical and chemical damage to lung tissue caused by meconium aspiration. *Pediatrics* 1978; 62(4): 454-459.

Vadász I, Morty RE, Kohstall MG, Olschewski A, Grimminger F, Seeger W, Ghofrani HA. Oleic acid inhibits alveolar fluid reabsorption: a role in acute respiratory distress syndrome? *Am J Respir Crit Care Med* 2005; 171(5): 469-479.

Vangerow B, Häfner D, Rueckoldt H, Marx G, Ott N, Leuwer M, Frerking I, Germann PG. Effects of C1 inhibitor and r-SP-C surfactant on oxygenation and histology in rats with lavage-induced acute lung injury. *Intensive Care Med* 2001; 27(9): 1526-1531.

Vidyasagar D, Zagariya A. Studies of meconium-induced lung injury: inflammatory cytokine expression and apoptosis. *J Perinatol* 2008; 28 Suppl 3: S102-107.

Villar J, Blanco J, Añón JM, Santos-Bouza A, Blanch L, Ambrós A, Gandía F, Carriedo D, Mosteiro F, Basaldúa S, Fernández RL, Kacmarek RM; ALIEN Network. The ALIEN study: incidence and outcome of acute respiratory distress syndrome in the era of lung protective ventilation. *Intensive Care Med* 2011; 37(12): 1932-1941.

Vlahakis NE, Schroeder MA, Limper AH, Hubmayr RD. Stretch induces cytokine release by alveolar epithelial cells in vitro. *Am J Physiol* 1999; 277(1 Pt 1): L167-173.

Wang HL, Akinci IO, Baker CM, Urich D, Bellmeyer A, Jain M, Chandel NS, Mutlu GM, Budinger GR. The intrinsic apoptotic pathway is required for lipopolysaccharide-induced lung endothelial cell death. *J Immunol* 2007; 179(3): 1834-1841.

Wang HM, Bodenstein M, Markstaller K. Overview of the pathology of three widely used animal models of acute lung injury. *Eur Surg Res* 2008; 40(4): 305-316.

Ware LB, Matthay MA. The acute respiratory distress syndrome. *N Engl J Med* 2000; 342(18): 1334-1349.

Welty-Wolf KE, Carraway MS, Ortel TL, Ghio AJ, Idell S, Egan J, Zhu X, Jiao JA, Wong HC, Piantadosi CA. Blockade of tissue factor-factor X

binding attenuates sepsis-induced respiratory and renal failure. *Am J Physiol Lung Cell Mol Physiol* 2006; 290(1): L21-31.

Wiener-Kronish JP, Albertine KH, Matthay MA. Differential responses of the endothelial and epithelial barriers of the lung in sheep to Escherichia coli endotoxin. *J Clin Invest* 1991; 88(3): 864-875.

Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* 1990; 249(4975): 1431-1433.

Zagariya A, Bhat R, Uhal B, Navale S, Freidine M, Vidyasagar D. Cell death and lung cell histology in meconium aspirated newborn rabbit lung. *Eur J Pediatr* 2000; 159(11): 819-826.

Zagariya A, Sierzputovska M, Navale S, Vidyasagar D. Role of meconium and hypoxia in meconium aspiration-induced lung injury in neonatal rabbits. *Mediators Inflamm* 2010; 2010: 204831.

Zarbock A, Singbartl K, Ley K. Complete reversal of acid-induced acute lung injury by blocking of platelet-neutrophil aggregation. *J Clin Invest* 2006; 116(12): 3211-3219.

Zila I, Mokra D, Kopincova J, Kolomaznik M, Javorka M, Calkovska A. Heart rate variability and inflammatory response in rats with lipopolysaccharide-induced endotoxemia. *Physiol Res* 2015; 64 Suppl 5: S669-676.

Zoban P, Cerny M. Immature lung and acute lung injury. *Physiol Res* 2003; 52(5): 507-516.

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Chapter 8

PROFESSIONAL TRAINING IN THE WORKUP OF BLEEDING DISORDERS IN WOMEN: OBSTETRICS-GYNECOLOGY RESIDENCY AND WOMEN'S HEALTH ADVANCED PRACTICE NURSING

Angelo P. Giardino[1], MD, PhD, Jennifer E. Dietrich[2], MD, MSc and Jennifer Kurkowski[3], MSN, WHNP

[1]Senior Vice President/Chief Quality Officer, Texas Children's Hospital, Houston, Texas; Professor and Section Head, Academic General Pediatrics, Baylor College of Medicine, Houston, Texas, US
[2]Division of Pediatric and Adolescent Gynecology, Department of Obstetrics and Gynecology, Baylor College of Medicine, Houston, Texas, US
[3]Division of Pediatric and Adolescent Gynecology, Department of Obstetrics and Gynecology, Baylor College of Medicine, Houston, Texas, US

ABSTRACT

More than 2 million women in the United States have an underlying bleeding disorder. The prevalence for bleeding disorders among women

with menorrhagia is 20%. The most commonly reported symptoms among individuals with a diagnosis of von Willebrand disease (VWD) or any suspected bleeding disorder include epistatxis, gingival bleeding, easy bruising and menorrhagia. Therefore, patient history is very important to guide an initial workup. Additionally, many recommend that the initial evaluation include complete blood count (CBC), prothrombin time (PT), activated partial thromboplastin time (PTT), international normalized ratio (INR), platelet function assay (PFA-100), blood type, bleeding time and VWF assays. There are a variety of treatments that exist for von Willebrand factor (VWF). The National Heart, Lung, and Blood (NHLBI) of the National Institutes of Health issued a guideline regarding bleeding disorders. This chapter describes the highlights of the NHLBI guidelines. In addition, the authors include information on the training of obstetrics-gynecology residents and Women's Health Advance Practice Nurses in the evaluation of bleeding disorders in women, and characterize the experience more fully and assess the evolving state of training in the evaluation of menorrhagia and bleeding disorders.

INTRODUCTION

Bleeding disorders are significant health challenges that affect nearly 3 million women per year in the United States. In this group of women, von Willebrand disease (VWD) is the most common bleeding disorder, affecting approximately 0.6-1.3% (American College of Obstetricians and Gynecologists, 2015) of women. As with any serious disorder, developing a consistent approach to the evaluation and management of the problem is optimal. This consistent approach ideally is broad enough to not miss cases but focused enough to avoid inefficient testing of patients unlikely to have the disorder.

A group of scientific experts convened in 2004 by the National Heart, Lung, and Blood Institute (NHLBI) addressed problems in the diagnosis, treatment, and health management of women with bleeding disorders (NHLBI, 2004). According to this NHLBI expert panel:

> Undiagnosed and untreated bleeding disorders in females have a negative impact on their health and quality of life. Because the most common bleeding disorders, including VWD, manifest primarily by mucosal bleeding, females are likely to be symptomatic due to excessive bleeding with menstruation (menorrhagia), ovulation, and childbirth. Studies have documented increased morbidity due to excessive bleeding

> in girls and women. Women with bleeding disorders are more likely to be iron deficient and to have received blood transfusions. They are also more likely to undergo gynecologic surgical procedures and to have bleeding complications related to those and other surgical procedures. Furthermore, a number of studies have shown the effect of this excessive bleeding, particularly menorrhagia, on quality of life, including increased symptoms of depression, and increased days lost from work and social activity. Since menorrhagia in women with bleeding disorders frequently begins at menarche, these factors could have an impact on the social and academic development of the adolescent female (NHLBI, 2004, p. 1).

The expert panel reached general agreement that a comprehensive bleeding history that helps guide laboratory testing is central to the clinical workup and diagnosis of bleeding disorders in women. In addition, the accurate classification of bleeding-related symptoms is essential along with an accurate, standardized quantification of the excessive bleeding. This requirement tends to be a clinical challenge since if frequently depends on self-report or a "surrogate measure" such as finding the presence of anemia in the patient being evaluated. The expert panel specifically addressed VWD, an inherited bleeding disorder "caused by a deficiency or dysfunction of von Willebrand factor (VWF), a plasma protein that mediates the initial adhesion of platelets at sites of vascular injury and also binds and stabilizes blood clotting factor VIII (FVIII) in the circulation" (NHLBI, 2004; 2007). As a result, planning for a guideline to inform the workup of bleeding disorders and especially VWD in women began in 2004; in 2007, the NHLBI issued treatment guidelines for VWD that addressed its diagnosis, evaluation, and management (described in further detail below) (NHLBI, 2007). Additionally, the Centers for Disease Control and Prevention (CDC) has identified a set of goals for the nation to achieve in its Healthy People 2020 objectives. Included are goals related to blood disorders and blood safety (BDBS), two of which address aspects of bleeding related to this chapter:

- BDBS-14: Increase the proportion of providers who refer women with symptoms suggestive of inherited bleeding disorders for diagnosis and treatment.
- BDBS-15: Increase the proportion of persons with VWD seen in specialty care centers who were diagnosed by 21 years of age

Goal BDBS-14 is currently under development. The CDC, in partnership with the American College of Obstetricians and Gynecologists (ACOG), plans to identify a nationally representative sample of obstetrician-gynecologists and evaluate their knowledge, attitudes, and practices related to the diagnosis of blood disorders. The survey process is underway and has the potential to be administered every 2-3 years. Goal BDBS-15 is currently operational with a 2012 baseline that 69.8% of persons with VWD who were seen in a specialty care center were diagnosed by 21 years of age. Figure 1 a-b displays the currently reported data related to Healthy People 2020's BDBS-15.

Disseminating the evidence-based practices contained in the 2007 NHLBI treatment guideline for VWD to clinicians who provide care to women involves many action steps, including the incorporation of the guideline's key components into the professional education of trainees. This chapter examines the clinical content of the guideline, the professional approach to teaching about bleeding disorders in women to ob-gyn residents and women's health advanced practice nurses, and survey data addressing the training in residencies and among advanced practice registered nurses (APRNs). It concludes with a brief discussion of why the adoption of clinical guidelines occurs via an academic process that takes time to unfold.

THE NHLBI VWD GUIDELINE

The NHLBI guidelines issued in 2007 addressed the diagnosis, evaluation, and management of VWD. The guidelines were evidence based, sought professional and public input, and were targeted towards practicing primary care and specialist clinicians including family physicians, internists, ob-gyns, pediatricians, nurse practitioners, hematologists, and laboratory medicine specialists.

VWD is a genetic disorder that affects the structure or function of the protein VWF, which is needed for normal blood clotting (Weiss, 2012). VWF works in the clot formation cascade by helping link platelets and subendothelium in an injured vessel wall as well as by transporting factor VIII, another protein that is important in clot stabilization (Weiss, 2012). Abnormalities in VWF can result in epitaxis, heavy menstrual bleeding, and

bleeding complications after trauma or surgery due to impaired platelet adhesion and decreased availability of factor VIII (Sadler, et al., 2006). VWD is classified by quantitative or qualitative defects in VWF, and there are three major types of VWD--types 1, 2, and 3. Type 2 is further categorized into type 2A, type 2B, type 2M, and type 2N. Type 1 is due to a quantitative defect and is usually characterized by mild to moderate bleeding symptoms. Type 2 VWD is a qualitative defect and is usually characterized by moderate to severe bleeding. The four subtypes of type 2 VWD are diagnosed by the type of VWF dysfunction. Finally, type 3 VWD is a rare quantitative defect characterized by severe bleeding symptoms due to absolute deficiency in VWF (Sadler et al., 2006). See Table X.1 a-b for further classification details.

Goal: BDBS-15 Increase the proportion of persons with von Willebrand disease (VWD) seen in specialty care centers who were diagnosed by 21 years of age

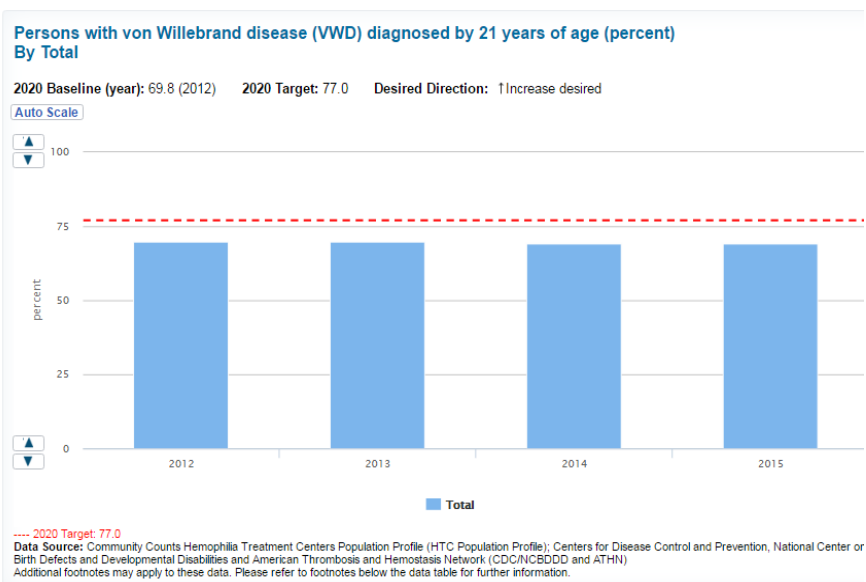
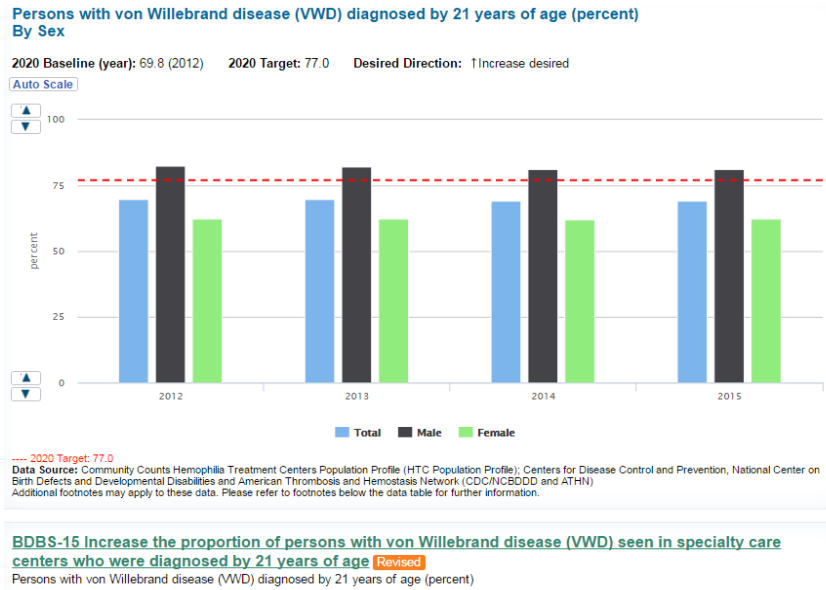


Figure 1a. Healthy People 2020's BDBS-15



(Healthy People 2020. (2016). Blood Disorders and Blood Safety)

Description of Changes since the Healthy People 2020 Launch: In 2015, this objective was revised from "Increase the proportion of women with von Willebrand disease (VWD) who are timely and accurately diagnosed" to "Increase the proportion of persons with von Willebrand disease (VWD) seen in specialty care centers who were diagnosed by 21 years of age." The baseline was revised from 28.4 percent (2008) to 70.0 percent (2012). The target was revised from 31.2 percent to 77.0 percent. The data source was revised from the Universal Data Collection (UDC) to Community Counts Hemophilia Treatment Center (HTC) Population Profile, CDC/NCBDDD. The target setting method remained 10 percent improvement over the baseline. In 2016, 2012-2014 data were revised due to reporting delays. This revision occurs annually. The baseline of the total population and target remained the same. Healthy People 2020. (2016). Blood Disorders and Blood Safety https://www.healthypeople.gov/2020/data-search/Search-the-Data#srch=persons with von Willebrand disease.

Figure 1b. Healthy People 2020's BDBS-15

Workup/Diagnosis

According to the NHLBI (2011), type 1 and type 2 VWD present with a range of mild to moderate bleeding symptoms including:

1. Frequent, large bruises from minor bumps or injuries.
2. Frequent or hard-to-stop nosebleeds.
3. Prolonged bleeding from the gums after a dental procedure.
4. Heavy or prolonged menstrual bleeding in women.
5. Blood in stools from bleeding in the intestines or stomach.
6. Blood in the urine from bleeding in the kidneys or bladder.
7. Heavy bleeding after a cut or other accident.
8. Heavy bleeding after surgery.

Type 3 VWD presents with the symptoms above and may have unexpected severe bleeding episodes as well (NHLBI, 2011). Type 3 VWD may also present with bleeding into soft tissues or joints, causing severe pain and swelling.

Among the most common presentations for women with VWD is menorrhagia or heavy menstrual bleeding characterized by:

- Bleeding with clots larger than about 1inch in diameter.
- The need to change pads or tampons more than every hour.
- Anemia.

Table 1a. Classification Details

Type	Description	Comments
1	Partial quantitative deficiency of VWF (70-75% of patients with VWD)	Type 1 VWD is found in persons who have partial quantitative deficiency of VWF. The level of VWF in plasma is low, and the remaining VWF mediates platelet adhesion normally and binds FVIII normally. Laboratory evaluation shows concordant decreases in VWF protein concentration (VWF:Ag) and assays of VWF function (VWF:RCo). Levels of blood clotting FVIII usually parallel VWF and may be reduced secondary to reduced VWF (p. 13)
2	Qualitative VWF defect (10-20% of patients with VWD)	The clinical features of several type 2 VWD variants are distinct from those of type 1 VWD, and they can have strikingly distinct and specific therapeutic needs. As a consequence, the medical care of patients who have type 2 VWD benefits from the participation of a hematologist who has expertise in hemostasis. Bleeding symptoms in type 2 VWD are often thought to be more severe than in type 1 VWD, although this impression needs to be evaluated in suitable clinical studies (p. 13)

Table 1a. (Continued)

Type	Description	Comments
2A	Decreased VWF-dependent platelet adhesion with selective deficiency of high-molecular-weight multimers	
2B	Increased affinity for platelet GPIb	
2M	Decreased VWF-dependent platelet adhesion without selective deficiency of high-molecular-weight multimers	
2N	Markedly decreased binding affinity for FVIII	
3	Virtually complete deficiency of VWF (rare)	Type 3 VWD is characterized by undetectable VWF protein and activity, and FVIII levels usually are very low (1–9 IU/dL). Nonsense and frameshift mutations commonly cause type 3 VWD, although large deletions, splice-site mutations, and missense mutations also can do so. Mutations are distributed throughout the VWF gene, and most are unique to the family in which they were first identified (p. 15)

Table 1b. Inheritance, Prevalence, and Bleeding Propensity in Patients Who Have VWD

Type	Inheritance	Prevalence	Bleeding Propensity
Type 1	Autosomal dominant	Up to 1%	Mild to moderate
Type 2A	Autosomal dominant (or recessive)	Uncommon	Variable—usually moderate
Type 2B	Autosomal dominant	Uncommon	Variable—usually moderate
Type 2M	Autosomal dominant (or recessive)	Uncommon	Variable—usually moderate
Type 2N	Autosomal recessive	Uncommon	Variable—usually moderate
Type 3 (Severe)	Autosomal recessive	Rare (1:250,000 to 1:1,000,000)	High (severe bleeding)

NHLBI, 2007; Weiss, J. (2012). Just heavy menses or something more? Raising awareness of von Willebrand disease. American Journal of Nursing, 112(6), 38-44.

Table 2 lists common bleeding symptoms in patients with VWD.

The medical history is central to the evaluation of bleeding disorders including VWD. (See Figure X.2.) According to the NHLBI (2011), the components of the ideal medical history for a potential bleeding disorder include:

- Any bleeding from a small wound that lasted more than 15 minutes or started up again within the first 7 days following the injury.
- Any prolonged, heavy, or repeated bleeding that required medical care after surgery or dental extractions.
- Any bruising with little or no apparent trauma, especially if a lump is felt under the bruise.
- Any nosebleeds that occurred for no known reason and lasted more than 10 minutes despite pressure on the nose, or any nosebleeds that needed medical attention.
- Any blood in stools for no known reason.
- Any heavy menstrual bleeding. This bleeding usually involves clots or lasts longer than 7 to 10 days.
- Any history of muscle or joint bleeding.
- Any medicines taken that might cause bleeding or increase the risk of bleeding. Examples include aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs), clopidogrel, warfarin, and heparin.
- Any history of liver or kidney disease, blood or bone marrow disease, or high or low blood platelet counts.

Table 2. Common Bleeding Symptoms of Healthy Individuals and Patients Who Have VWD

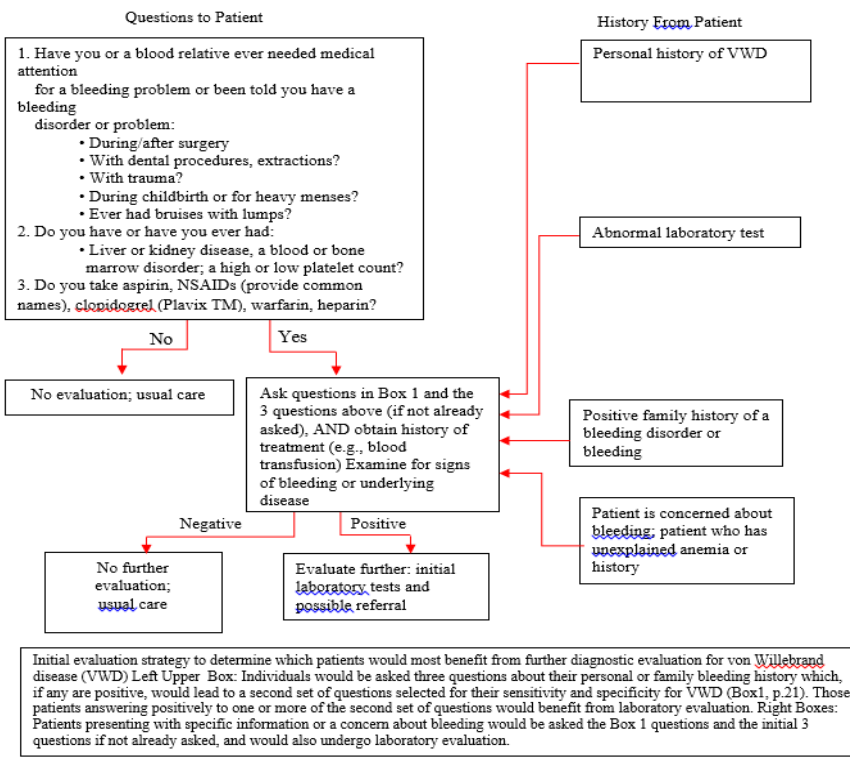
Symptoms	Normals (n = 500;[137] n= 341;‡[138] n = 88;‡‡[139] n= 60‡‡[140]) %	All types VWD (n = 264;[137] n = 1,885[141]) %	Type 1 VWD (n = 42;†[142] n = 671[136]) %	Type 2 VWD (n = 497[136]) %	Type 3 VWD (n = 66;[136] n = 385[85]) %
Epistaxis	4.6–22.7	38.1–62.5	53–61	63	66–77
Menorrhagia*	23–68.4	47–60	32	32	56–69
Bleeding after dental extraction	4.8–41.9	28.6–51.5	17–31	39	53–70
Ecchymoses	11.8–50	49.2–50.4	50	N.R.	N.R.
Bleeding from minor cuts or abrasions	0.2–33.3	36	36	40	50
Gingival bleeding	7.4–47.1	26.1–34.8	29–31	35	56
Postoperative bleeding	1.4–28.2	19.5–28	20–47	23	41
Hemarthrosis	0–14.9	6.3–8.3	2–3	4	37–45
Gastrointestinal bleeding	0.6–27.7	14	5	8	20

* Calculated for females above 13 to 15 years of age.
‡ 341 individuals were sent a questionnaire, but the precise number of patients responding was not provided.
‡‡ Study included women only.
† Study included males only.
N.R., Not reported.

(National Heart, Lung, and Blood Institute, 2007. p. 21.)

The physical examination focuses on the identification of bruising and signs of anemia, such as pallor, and vital sign changes. Of course, the laboratory evaluation hinges on appropriate blood tests based on what the medical history and the physical examination suggest. If VWD is suspected, the NHLBI (2001) suggests the following tests:

- VWF antigen.
- VWF ristocetin cofactor activity.
- Factor VIII clotting activity. This test checks the clotting activity of factor VIII.
- VWF multimers (typically ordered if first three tests are abnormal).
- Platelet function test.

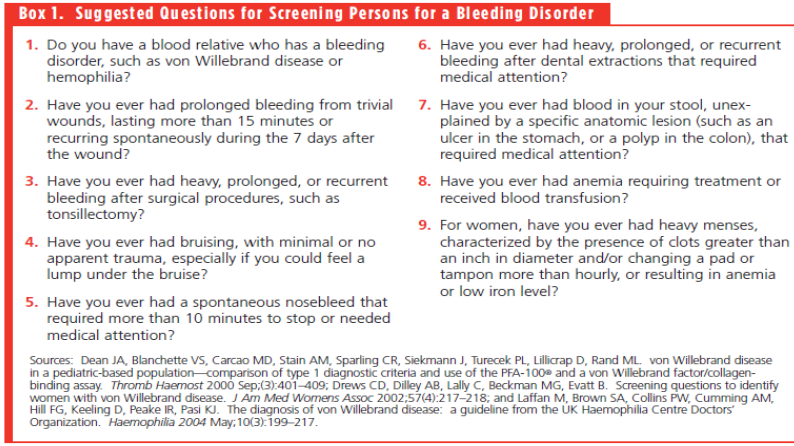


(National Heart, Lung, and Blood Institute, 2007).

Figure 2. The NHLBIVWD treatment guideline suggests this algorithm

The laboratory evaluation guided by the medical history and physical examination is further described in Figure 3.

Box 1. Suggested Questions for Screening Persons for a Bleeding Disorder



Box 1. Suggested Questions for Screening Persons for a Bleeding Disorder

1. Do you have a blood relative who has a bleeding disorder, such as von Willebrand disease or hemophilia?
2. Have you ever had prolonged bleeding from trivial wounds, lasting more than 15 minutes or recurring spontaneously during the 7 days after the wound?
3. Have you ever had heavy, prolonged, or recurrent bleeding after surgical procedures, such as tonsillectomy?
4. Have you ever had bruising, with minimal or no apparent trauma, especially if you could feel a lump under the bruise?
5. Have you ever had a spontaneous nosebleed that required more than 10 minutes to stop or needed medical attention?
6. Have you ever had heavy, prolonged, or recurrent bleeding after dental extractions that required medical attention?
7. Have you ever had blood in your stool, unexplained by a specific anatomic lesion (such as an ulcer in the stomach, or a polyp in the colon), that required medical attention?
8. Have you ever had anemia requiring treatment or received blood transfusion?
9. For women, have you ever had heavy menses, characterized by the presence of clots greater than an inch in diameter and/or changing a pad or tampon more than hourly, or resulting in anemia or low iron level?

Sources: Dean JA, Blanchette VS, Carcao MD, Stain AM, Sparling CR, Siekmann J, Turecek PL, Lillicrap D, Rand ML. von Willebrand disease in a pediatric-based population—comparison of type 1 diagnostic criteria and use of the PFA-100® and a von Willebrand factor/collagen-binding assay. *Thromb Haemost* 2000 Sep;(3):401–409; Drews CD, Dilley AB, Lally C, Beckman MG, Evatt B. Screening questions to identify women with von Willebrand disease. *J Am Med Womens Assoc* 2002;57(4):217–218; and Laffan M, Brown SA, Collins PW, Cumming AM, Hill FG, Keeling D, Peake IR, Pasi KJ. The diagnosis of von Willebrand disease: a guideline from the UK Haemophilia Centre Doctors' Organization. *Haemophilia 2004* May;10(3):199–217.

Source: Dean JA, Blanchette VS, Carcao MD, Stein AM, Sparling CR, Siekmann J, Turecek PL, Lillicrap D, Rand ML. von Willebrand disease in a pediatric-based population – comparison of type 1 diagnostic criteria and use of the PFA-100® and a von Willebrand factor/collagen-binding assay. *Thromb. Haemost 2000* Sep;(3):401-409; Drews CD, Dilley AB, Lally C, Beckman MG, Evatt B. Screening questions to identify women with von Willebrand disease. *J. Am. Med. Womens Assoc*. 2002;57(4):2017-2018; and Laffan M, Brown SA, Collins PW, Cumming AM, Hill FG, Keeling D, Peake IR, Pasi KJ. The diagnosos of von Willebrand disease: a guideline from the UK Haemophillia Centre Doctors' Organization. *Haemophilia* 2004 May;10(3):199-217.

(National Heart, Lung, and Blood Institute, 2007. pp. 20-21.)

Additionally, the NHLBI (2007) VWD guidelines contain recommendations on specific clinical history, physical findings, laboratory assays, and diagnostic criteria that allow for the most accurate diagnosis of VWD:

- Tests such as the bleeding time, PFA-100, or other automated functional platelet assays have been used, but there are conflicting data with regard to sensitivity and specificity for VWD. Therefore, the Panel believes current evidence does not support their routine use as screening tests for VWD.
- The Panel believes that platelet-based assays should be used for the ristocetin cofactor method.
- The Panel emphasizes the importance of the timing of the phlebotomy for assays, with the patient at his/her optimal baseline as far as

possible (For example, VWF levels maybe elevated above baseline during the second and third trimesters of pregnancy or during estrogen replacement, during acute inflammation such as the perioperative period during infections, and during acute stress). The careful handling and processing of the sample is also critical, particularly if the sample will be sent out for testing at a distant location (NHLBI, 2007. p. 33).

Treatment

According to NHLBI (2011), the treatment for VWD is based on the type of VWD and its clinical severity. (See Table 3.) Mild cases of VWD may only need treatment when they involve surgical procedures or tooth extractions, or in the setting of trauma. (See Figure 3.)

Medications used are directed at:

- Increasing the amount of circulating VWF and factor VIII.
- Replacing VWF.
- Preventing the breakdown of blood clots.
- Controlling heavy menstrual bleeding.

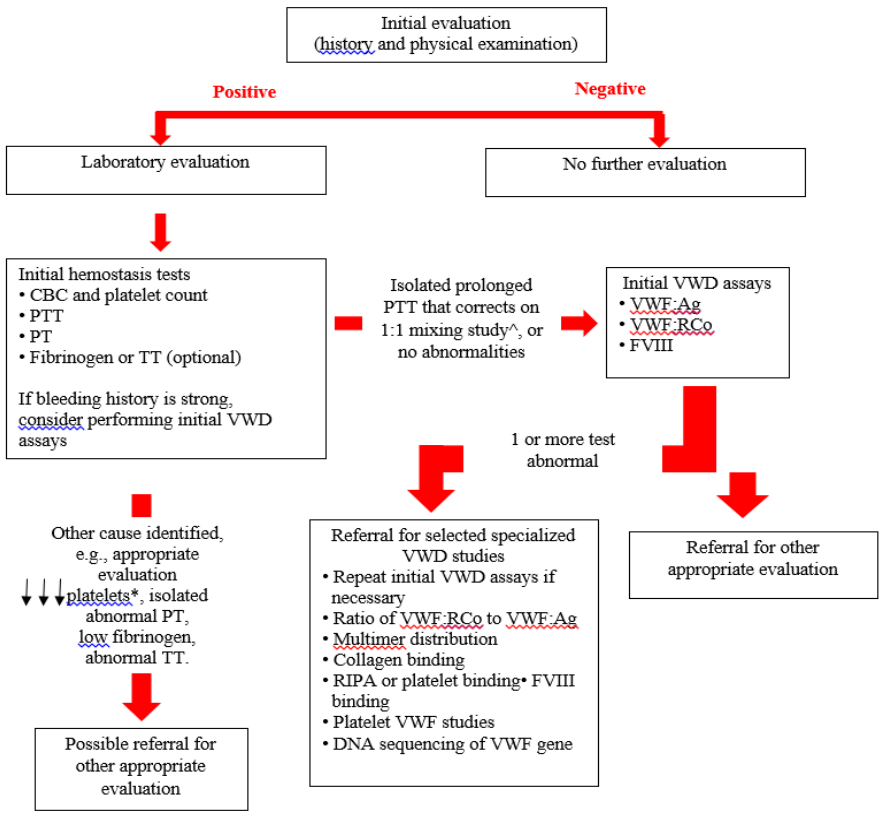
These medications include desmopressin either injected or administered via nasal spray cause the release of VWF and factor VIII into the bloodstream. Desmopressin works well for those persons with type 1 VWD and for some who have type 2 VWD.

Replacement therapy for VWD involves an infusion of concentrated VWF, and factor VIII may be used for:

- Those who don't tolerate desmopressin or who need extended treatment.
- Type 1 VWD that does not respond to desmopressin.
- Type 2 or type 3 VWD.

Antifibrinolytic medicines also are used to treat VWD and help prevent the breakdown of blood clots. These medications are used to stop bleeding

after minor surgery, tooth extraction, or an injury, and may be used alone or in combination with desmopressin and replacement therapy.



* Isolated decreased platelets may occur in VWD type 2B.

^ Correction in the PTT mixing study immediately and after 2-hour incubation removes a factor VIII (FVIII) inhibitor from consideration.

Investigation of other intrinsic factors and lupus anticoagulant also may be indicated.

CBC, complete blood count; PT prothrombin time; PTT partial thromboplastin time; RIPA, ristocetin-induced platelet aggregation; TT, thrombin time; VWF:Ag, VWF antigen; VWF:RCo, VWF ristocetin cofactor activity.

If the initial clinical evaluation suggests a bleeding disorder, the "initial hemostasis tests" should be ordered, followed by or along with the next tests ("initial VWD assays") indicated in the algorithm. Referral to a hemostasis specialist is appropriate for help in interpretation, repeat testing, and specialized tests.

(National Heart, Lung, and Blood Institute, 2007. p. 25.)

Figure 3. Laboratory Assessment for VWD or Other Bleeding Disorder

Table 3. Laboratory Values from Which to Interpret Patient Results

Condition	VWF:RCo (IU/dL)	VWF:Ag (IU/dL)	FVIII	Ratio of VWF:RCo/ VWF:Ag
Type 1	<30*	<30*	↓or Normal	>0.5–0.7
Type 2A	<30*	<30–200*†	↓or Normal	<0.5–0.7
Type 2B	<30*	<30–200*†	↓or Normal	Usually <0.5–0.7
Type 2M	<30*	<30–200*†	↓or Normal	<0.5–0.7
Type 2N	30–200	30–200	↓↓	>0.5–0.7
Type 3	<3	<3	↓↓↓ (<10 IU/dL)	Not applicable
"Low VWF"	30–50	30–50	Normal	>0.5–0.7
Normal	50–200	50–200	Normal	>0.5–0.7

↓ Refers to a decrease in the test result compared to the laboratory reference range.

* <30 IU/dL is designated as the level for a definitive diagnosis of VWD: there are some patients with type 1 or type 2 VWD who have levels of VWF:RCo and/or VWF:Ag of 30-50 IU/dL.

+ The VWF:Ag in the majority of individuals with type 2A, 2B, or 2M VWD is <50 IU/dL.

(National Heart, Lung, and Blood Institute, 2007. p. 36.)

For women who have VWD and experience heavy menstrual bleeding, additional options include:

- Oral contraceptives, which can increase the amount of circulating VWF and factor VIII and can reduce menstrual blood loss.
- An intrauterine device containing levonorgestrel, a progestin hormone.
- Aminocaproic acid or tranexamic acid, antifibrinolytic medications that reduce bleeding by slowing the breakdown of blood clots.
- Desmopressin.

For women beyond child-bearing age, endometrial ablation, which destroys the lining of the uterus, may reduce menstrual blood loss in those who have VWD. Of course, if a woman undergoes a hysterectomy, the uterus is surgically removed, which anatomically stops menstrual bleeding.

PROFESSIONAL EDUCATION REGARDING THE CLINICAL WORKUP IN BLEEDING DISORDERS

Improving women's health curricula can promote advances in the field. Toward that goal, the Health Resources and Services Administration (HRSA) Office of Women's Health (US Department of Health and Human Services, 2013) commissioned a report to provide the background, recommendations, and implementation steps to improve women's health education across five specific health profession programs: medicine, oral health/dentistry, baccalaureate nursing, pharmacy, and public health. Key content areas in women's health across the health professions include wellness and prevention, biological considerations, selected conditions, behavioral health, and the role of the health professional. Figure 4 a-b array the approach to this collaborative effort.

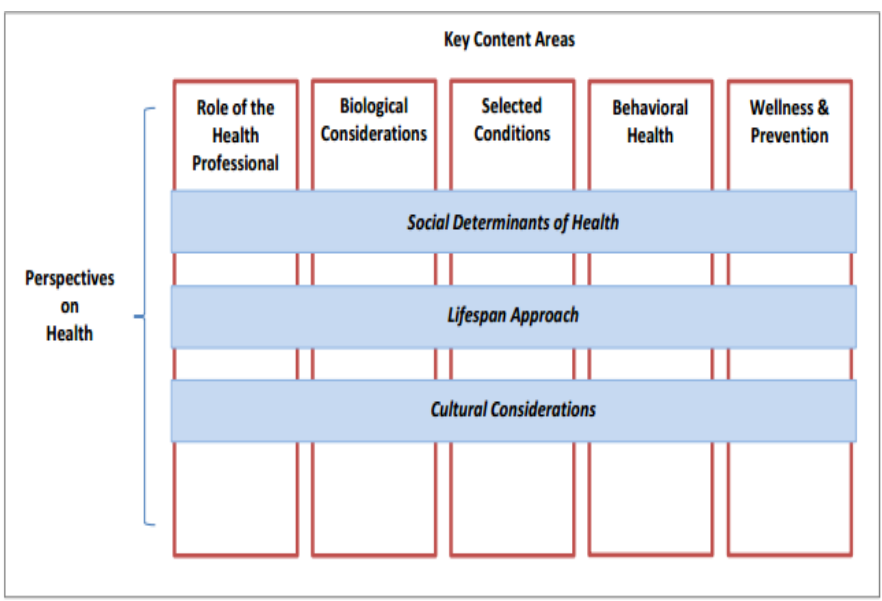
Obstetrics-Gynecology Residents

Obstetrics-gynecology is the physician specialty in which training and practice focus on the conditions and disorders related to female reproductive organs and the management of pregnancy and delivery. ACOG's Council on Resident Education in Obstetrics and Gynecology (CREOG) annually publishes goals for residents in a document titled "Educational Objectives: Core Curriculum in Obstetrics and Gynecology" (American College of Obstetrics and Gynecology, 2013). It follows six general competencies mandated by the Accreditation Council for Graduate Medical Education (ACGME):

- Patient care
- Medical knowledge
- Interpersonal and communication skills
- Professionalism
- Practice-based learning and improvement
- System-based practice

The 11th edition of CREOG's Educational Objectives, released in 2016, addresses dysmenorrhea and abnormal uterine bleeding in Unit 4 (Gynecologic Disorders) and covers dysmenorrhea in Unit 5 (Pediatric and Adolescent Obstetrics and Gynecology). Additionally, in the prior 10th edition, released in 2013 in a slightly more detailed fashion, the evaluation of menstrual disorders is covered in Unit 4 (Gynecology) and Unit 5 (Reproductive Endocrinology and Infertility). Within Unit 4 is a section focusing on the evaluation and management of abnormal uterine bleeding or dysfunctional uterine bleeding, which states that a resident should be able to elicit pertinent history to evaluate causes of abnormal uterine bleeding and to appropriately interpret a complete blood count as well as coagulation tests (American College of Obstetricians and Gynecologists, 2013).

In Unit 5, under the subsection Pediatric and Adolescent Gynecology, the evaluation of menstrual and endocrine disorders refers back to Unit 4. The objectives do not contain specific language regarding the consistent evaluation for bleeding disorders in women and adolescents (American College of Obstetricians and Gynecologists, 2013). Table 4 arrays these objectives and competencies.



(U.S. Department of Health and Human Services, 2013, p. 11)
Figure 4a. Conceptual Approach to Inter-professional Women's Health Content.

Common Content Areas in Women's Health Across the Health Professions

Area	Sample Topics
Role of the Health Professional	• Ethics • Interprofessional Education • Knowledge of Other Health Professions • Patient-centered Decision-making • Gender in Provider/Patient Communication
Biological Considerations	• Age • Sex • Genetics • Hormonal Influences • Pharmacokinetics and Pharmacodynamics
Selected Conditions	• Autoimmune Disorders • Cardiovascular Disease • Endocrine Disorders • Endometriosis • Infectious Disease (Especially HIV) • Pregnancy and Breastfeeding (Especially medications taken during pregnancy and periodontal health in pregnancy) • Metabolic Disorders • Musculoskeletal Health • Neurological Conditions
Behavioral and Mental Health	• Anxiety/Stress • Depression/Bipolar Disorders • Domestic/Intimate Partner Violence • Eating Behaviors/Disorders • Sexual Behavior • Substance Abuse • Traumatic Experiences
Wellness and Prevention	• Access to Care • Environmental Health • Exercise Physiology • Hormonal Transitions • Nutrition • Oral Health • Reproductive Choice, Family Planning, and Obstetrics • Preventative Health Screening and Immunizations • Work-Family Balance

(U.S. Department of Health and Human Services, 2013, p. 14)

Figure 4b. Approach to Improving Education on Women's Health.

Table 4. Objectives and Competencies for Ob-Gyn Residency Training

CREOG Objective	Descriptions	ACGME Competency
Unit 4 - Gynecology Section II - Disorders Of The Urogenital Tract And Breast	A. Abnormal uterine bleeding	
	1. Describe the principal causes of abnormal uterine bleeding and the International Federation of Gynecology and Obstetrics (FIGO) classification system.	Medical Knowledge
	2. Obtain a pertinent history to evaluate abnormal uterine bleeding.	Patient Care
	3. Perform a focused physical examination to investigate the etiology of abnormal uterine bleeding.	

Table 4. (Continued)

CREOG Objective	Descriptions	ACGME Competency
	4. Perform and interpret the results of selected diagnostic tests, such as the following, to determine the cause of abnormal uterine bleeding: a. Endometrial biopsy b. Pelvic ultrasonography/saline infusion ultrasonography c. Hysteroscopy d. Laparoscopy	
	5. Interpret the results of other diagnostic tests, such as the following: a. Serum/urine human chorionic gonadotropin (hCG) assay b. Endocrinologic assays c. Microbiologic cultures of the genital tract d. Complete blood count e. Coagulation profile	
	6. Treat abnormal uterine bleeding using both nonsurgical and surgical methods.	
	7. Recommend appropriate follow-up that is necessary for a patient with abnormal uterine bleeding.	
Unit 5 III. Menstrual And Endocrine Disorders	A. Dysmenorrhea	
	Describe the classification of dysmenorrhea (i.e., primary versus secondary).	Medical Knowledge
	List the principal causes of primary and secondary dysmenorrhea.	Medical Knowledge
	Obtain a pertinent history to evaluate dysmenorrhea.	Interpersonal and Communication Skills
	Perform a focused physical examination to evaluate dysmenorrhea.	Patient Care
Unit 5 III. Menstrual And Endocrine Disorders	Perform and/or interpret indicated tests to evaluate dysmenorrhea	Patient Care
	Describe medical and surgical treatment options for dysmenorrhea	
	Describe long term follow-up and prognosis for a patient with dysmenorrhea, especially regarding reproduction and sexual function.	
	Endometriosis and adenomyosis (covered in Unit 4, II-J),	
	B. Abnormal Uterine Bleeding	
	Abnormal uterine bleeding (see above, Unit 4, II-A)	

(The American College of Obstetricians and Gynecologists, 2013.) (Used with permission)

Women's Health Advanced Practice Nurses

Women's Health Nurse Practitioners are advanced practice nurses who provide primary and specialty care to women throughout their life course. Core competencies are defined for all entry-level nurse practitioners (NPs) and are supplemented by the specialty-specific competencies (population-focused competencies) for the NPs who focus on women's health as a specialty area (National Organization of Nurse Practitioners Faculties [NONPF]), 2012; 2014). The development of the competencies is evolutionary and involves a national consensus process that responds to emerging evidence and health care delivery needs.

In 2002, HRSA supported an effort co-facilitated by NONPF and the American Association of Colleges of Nursing (AACN) and published a comprehensive set of core and specialty-specific competencies covering the following seven domains (US Department of Health and Human Services, 2002):

- Management of Patient Health/Illness Status
- The Nurse Practitioner-Patient Relationship
- The Teaching-Coaching Function
- Professional Role
- Managing and Negotiating Health Care Delivery Systems
- Monitoring and Ensuring the Quality of Health Care Practice
- Cultural Competence

In this document the specialty competencies contained a separate section titled "Diagnosis of Health Status" which emphasized that nurse practitioners are engaged in the diagnostic process, including critical thinking involved in differential diagnosis and the integration and interpretation of various forms of disorders. So, regarding women's health providers, bleeding disorders were not specifically called out in either the core or specialty NP competencies, but one would expect this clinical topic to be addressed as scientific knowledge increased in this area, i.e., with the release of the 2007 NHLBI VWD guidelines. Table 5 lists the core competencies in the Management of Patient Health/Illness Status, along with the specialty supplement for the women's health population focus, as articulated in the 2002 HRSA document.

Table 5. Selected Domains and Competency for NPs

Domain 1: Management of Patient Health/Illness Status	
Core Competency for all NPs	Women's Health Specialty Specific
The nurse practitioner demonstrates competence in the domain of management of patient health/illness status when she or he performs the following behaviors in the following areas. *A. Health Promotion/Health Protection and Disease Prevention* 1. Differentiates between normal, variations of normal, and abnormal findings. 2. Provides health promotion and disease prevention services to patients who are healthy or have acute and chronic conditions, based on age, developmental stage, family history, and ethnicity.	The women's health nurse practitioner is a provider of direct health care services. Within this role, the women's health nurse practitioner synthesizes theoretical, scientific, and contemporary clinical knowledge for the assessment and management of both health and illness states. These competencies incorporate the health promotion, health protection, disease prevention, and treatment focus of women's health nurse practitioner practice.
3. Provides anticipatory guidance and counseling to promote health, reduce risk factors, and prevent disease and disability, based on age, developmental stage, family history, and ethnicity. 4. Develops or uses a follow-up system within the practice to ensure that patients receive appropriate services.	*A. Assessment of Health Status* These competencies describe the role of the women's health nurse practitioner in assessing all aspects of the patient's health status, including for purposes of health promotion, health protection, and disease prevention.
Domain 1: Management of Patient Health/Illness Status	
Core Competency for all NPs	Women's Health Specialty Specific

5. Recognizes environmental health problems affecting patients and provides health protection interventions that promote healthy environments for individuals, families, and communities. *B. Management of Patient Illness* 1. Analyzes and interprets history, including presenting symptoms, physical findings, and diagnostic information to develop appropriate differential diagnoses. 2. Diagnoses and manages acute and chronic conditions while attending to the patient's response to the illness experience. 3. Prioritizes health problems and intervenes appropriately including initiation of effective emergency care. 4. Employs appropriate diagnostic and therapeutic interventions and regimens with attention to safety, cost, invasiveness, simplicity, acceptability, adherence, and efficacy. 5. Formulates an action plan based on scientific rationale, evidence-based standards of care, and practice guidelines. 6. Provides guidance and counseling regarding management of the health/illness condition. 7. Initiates appropriate and timely consultation and/or referral when the problem exceeds the nurse practitioner's scope of practice and/or expertise. 8. Assesses and intervenes to assist the patient in complex, urgent, or emergency situations	The women's health nurse practitioner employs evidence-based clinical practice guidelines to guide screening activities, identifies health promotion needs, and provides anticipatory guidance and counseling addressing environmental, lifestyle, and developmental issues. 1. Obtains and documents a relevant health history, including a comprehensive obstetric and gynecologic history, with emphasis on gender-based differences. 2. Performs and documents complete, system, or symptom-directed physical examinations on women, including obstetric and gynecologic conditions/needs that include, but are not limited to, pregnancy, benign and malignant gynecologic conditions, contraception, sexually transmitted infections, infertility, perimenopause/menopause/postmenopause and other gender-specific illnesses. 3. Assesses for maternal and fetal well-being, high-risk pregnancies, depression, and pregnancy/post-partum complications. 4. Assesses for disease risk factors specific to women. 5. Distinguishes female gender differences in presentation and progression of health problems and responses to pharmacological agents and other therapies. 6. Assesses social and physical environmental health risks, including teratogens, that impact childbearing. 7. Assesses for evidence of domestic violence, sexual abuse, and substance abuse. 8. Assesses issues related to sexuality.

Table 5. (Continued)

a. Assesses rapidly the patient's unstable and complex health care problems through synthesis and prioritization of historical and immediately derived data.	9. Assesses parental behavior and skills and promotes smooth transition to role changes.
Domain 1: Management of Patient Health/Illness Status	
Core Competency for all NPs	Women's Health Specialty Specific
b. Diagnoses unstable and complex health care problems utilizing collaboration and consultation with the multidisciplinary health care team as indicated by setting, specialty, and individual knowledge and experience, such as patient and family risk for violence, abuse, and addictive behaviors. c. Plans and implements diagnostic strategies and therapeutic interventions to help patients with unstable and complex health care problems regain stability and restore health in collaboration with the patient and multidisciplinary health care team. d. Rapidly and continuously evaluates the patient's changing condition and response to therapeutic interventions, and modifies the plan of care for optimal patient outcomes. *Appropriate to Both Subdomains* 1. Demonstrates critical thinking and diagnostic reasoning skills in clinical decision making. 2. Obtains a comprehensive and problem-focused health history from the patient. 3. Performs a comprehensive and problem-focused physical examination. 4. Analyzes the data collected to determine health status. 5. Formulates a problem list. 6. Assesses, diagnoses, monitors, coordinates, and manages the health/illness status of patients over time and supports the patient through the dying process. 7. Demonstrates knowledge of the pathophysiology of acute and chronic diseases or conditions commonly seen in practice.	10. Assesses selected reproductive health needs or problems in male partners, such as sexually transmitted infections, contraception, and infertility. 11. Assesses genetic risks and refers, as needed, for testing and counseling. *B. Diagnosis of Health Status* The women's health nurse practitioner is engaged in the diagnosis of health status. This diagnostic process includes critical thinking, differential diagnosis, and the integration and interpretation of various forms of data. These competencies describe this role of the women's health nurse practitioner. 1. Diagnoses common non-gynecologic health problems and other deviations from normal and provides management, education, or referral when appropriate. 2. Identifies obstetrical and gynecologic deviations from normal, formulates a diagnosis, collaborates, and/or refers as necessary. 3. Performs and interprets screening and diagnostic procedures, including, but not limited to, pap tests, microscopy, post coital tests, and sexually transmitted infection tests. 4. Orders screening and diagnostic procedures and interprets test results, including, but not limited to, ultrasound, mammography, endometrial biopsies, colposcopy, triple screen, and fetal assessment tests, as well as age appropriate primary care screens.

8. Communicates the patient's health status using appropriate terminology, format, and technology.	5. Diagnoses acute and chronic conditions with an emphasis on reproductive/gynecologic health, including, but not limited to, pregnancy, sexually transmitted infections, infertility, benign and malignant gynecologic conditions, peri- and postmenopause, and other gender-specific conditions.
Domain 1: Management of Patient Health/Illness Status	
Core Competency for all NPs	Women's Health Specialty Specific
9. Applies principles of epidemiology and demography in clinical practice by recognizing populations at risk, patterns of disease, and effectiveness of prevention and intervention.	
10. Uses community/public health assessment information in evaluating patient needs, initiating referrals, coordinating care, and program planning. 42 11. Applies theories to guide practice. 12. Applies/conducts research studies pertinent to area of practice. 13. Prescribes medications based on efficacy, safety, and cost as legally authorized and counsels concerning drug regimens, drug side effects, and interactions with food supplements and other drugs. 14. Integrates knowledge of pharmacokinetic processes of absorption, distribution, metabolism, and excretion, and factors that alter pharmacokinetics in drug dosage and route selection. 15. Selects/prescribes correct dosages, routes, and frequencies of medications based on relevant individual patient characteristics, e.g., illness, age, culture, gender, and illness. 16. Detects and minimizes adverse drug reactions with knowledge of pharmacokinetics and dynamics with special attention to vulnerable populations such as infants, children, pregnant and lactating women, and older adults. 17. Evaluates and counsels the patient on the use of complementary/alternative therapies for safety and potential interactions.	6. Recognizes the importance of specimen collection and preservation in obtaining forensic evidence in victims of sexual assault and refers for further evaluation. 7. Diagnoses selected conditions related to the male reproductive system, such as sexually transmitted infections, contraceptive needs, and infertility. *C. Plan of Care and Implementation of Treatment* The objectives of planning and implementing therapeutic interventions are to return the patient to a stable state and to optimize the patient's health. These competencies describe the women's health nurse practitioner's role in stabilizing the patient, minimizing physical and psychological complications, and maximizing the patient's health potential. 1. Provides health promotion and disease prevention services to women across the life cycle, taking into account age, developmental status, disability, culture, ethnicity, sexual orientation, spiritual/religious affiliation, and lifestyle and psychosocial issues. 2. Provides prenatal and postnatal care including, but not limited to, maternal/fetal health, parent/infant relationships, lactation and parenting skills.

Table 5. (Continued)

18. Integrates appropriate nonpharmacologic treatment modalities into a plan of management.	3. Collaborates with other health care providers for management or referral of high-risk pregnancies.
Domain 1: Management of Patient Health/Illness Status	
Core Competency for all NPs	Women's Health Specialty Specific
19. Orders, may perform, and interprets common screening and diagnostic tests. 20. Evaluates results of interventions using accepted outcome criteria, revises the plan accordingly, and consults/refers when needed. 21. Collaborates with other health professionals and agencies as appropriate.	4. Provides anticipatory guidance and counseling to pregnant women and their significant others. 5. Treats women for selected obstetric and gynecologic problems/needs, including, but not limited to, pregnancy, common gynecologic conditions, contraception, sexually transmitted infections, peri- and postmenopause, and other gender specific illnesses.
22. Schedules follow-up visits to appropriately monitor patients and evaluate health/illness care.	6. Provides management and education for women and men in need of family planning and fertility control. 7. Manages the treatment of sexually transmitted infections for patients and their partners. 8. Formulates and implements a plan of care for women in violent/abusive relationships and victims of sexual assault, and considers legal reporting guidelines. 9. Treats men with selected reproductive health needs or problems, such as contraception and sexually transmitted infections. 10. Manages and/or refers for primary care conditions, including, but not limited to, headaches, hypertension, urinary tract infections, upper respiratory infections, and common dermatological conditions. 11. Performs primary care procedures, including, but not limited to, pap smears, microscopy, post-coital tests, intrauterine device (IUD) insertion, and endometrial biopsies. 12. Prescribes therapies, including medications, considering

	pregnancy, lactation, sociocultural background, and financial resources. 13. Applies theories from the social sciences and humanities, as well as natural sciences and nursing, including feminist and culturally relevant frameworks. 14. Applies research that is women-centered and contributes to positive change in the health of women or the health care delivered to women. 15. Facilitates access to reproductive health care services and provides referrals that are provided in an unbiased, timely, and sensitive manner.

(The US Department of Health and Human Services, 2002.)

The domains, or competency areas, have continued to be updated and enhanced in the ensuing years, and via national workgroups the National Organization of Nurse Practitioner Faculties (NONPF) suggests content for the core and population-focused competencies along with supportive curricular approaches (2012; 2013; 2014; 2016). The current 9 competency areas are:

- Scientific Foundation
- Leadership
- Quality
- Practice Inquiry
- Technology and Information Literacy
- Policy
- Health Delivery System
- Ethics
- Independent Practice

Again, as in the 2002 version of core and specialty competencies, while the topic of bleeding disorders is not specifically addressed, this clinical topic would be addressed in both the scientific foundation and independent practice competency areas dealing with the incorporation of evidence-based standards into one's practice. Table X.6 a-d provides a summary of the core and population-focused competencies that would be most applicable to evaluation and management of bleeding disorders in women.

PROFESSIONAL TRAINING SURVEYS

To assess the educational approach to bleeding disorder evaluation in ob-gyn residency training programs as well as among Women's Health Advanced Practice Nurses in the continental United States, two surveys were conducted, one with ob-gyn chief residents and the other with practicing Women's Health NPs. The surveys covered training experiences and fund of knowledge regarding the evaluation of menorrhagia and diagnosis of bleeding disorders during training.

Table 6. Competencies Most Applicable to Evaluation and Management of Bleeding Disorders in Women

Table 6a. Compentency Area Scientific Foundation

Competency Area	NP Core Competencies	Curriculum Content to Support Competencies *Neither required nor comprehensive, this list reflects only suggested content specific to the core competencies*
Scientific Foundation Competencies	1. Critically analyzes data and evidence for improving advanced nursing practice. 2. Integrates knowledge from the humanities and sciences within the context of nursing science. 3. Translates research and other forms of knowledge to improve practice processes and outcomes. 4. Develops new practice approaches based on the integration of research, theory, and practice knowledge.	Comparison of patient data sets with evidence-based standards to improve care Scientific foundations to practice, including, but not limited to, knowledge of advanced pathophysiology, pharmacology, physiology, genetics, and communication skills Science from other disciplines relevant to health care

(The National Organization of Nurse Practitioners Faculties [NONPF], 2014, p. 2) (Used with permission)

Table 6b. Population Focused: Women's Health/Gender Related

Competency Area	NP Core Competencies	Women's Health / Gender-Related NP Competencies	Curriculum Content to Support Competencies *Neither required nor comprehensive, this list reflects only suggested content specific to the population*
Scientific Foundation Competencies	1. Critically analyzes data and evidence for improving advanced nursing practice. 2. Integrates knowledge from the humanities and sciences within the context of nursing science. 3. Translates research and other forms of knowledge to improve practice processes and outcomes. 4. 4. Develops new practice approaches based on the integration of research, theory, and practice knowledge	1. Integrates research, theory, and evidence-based practice knowledge to develop clinical approaches that address women's responses to physical and mental health and illness across the lifespan. 2. Integrates best evidence into practice incorporating client values and clinical judgment	Hormonal therapy (contraception, HRT, infertility/fertility treatments) In-depth knowledge of reproductive endocrinology Advanced assessment of female breast and genitourinary systems Genomics Advanced practice and interprofessional role development Gender discrimination Sexual Assault Gender-unique disease presentations

(The National Organization of Nurse Practitioners Faculties [NONPF], 2013, p. 78)
(Used with permission)

Table 6c. Independent Practice Competencies

Competency Area	NP Core Competencies	Curriculum Content to Support Competencies *Neither required nor comprehensive, this list reflects only suggested content specific to the core competencies*
Independent Practice Competencies	1. Functions as a licensed independent practitioner. 2. Demonstrates the highest level of accountability for professional practice. 3. Practices independently managing previously diagnosed and undiagnosed patients. 3.a Provides the full spectrum of health care services to include health promotion, disease prevention, health protection, anticipatory guidance, counseling, disease management, palliative, and end-of-life care. 3.b Uses advanced health assessment skills to differentiate between normal, variations of normal and abnormal findings. 3.c Employs screening and diagnostic strategies in the development of diagnoses. 3.d Prescribes medications within scope of practice. 3.e Manages the health/illness status of patients and families over time. 4. Provides patient-centered care recognizing cultural diversity and the patient or designee as a full partner in decision-making. 4.a Works to establish a relationship with the patient characterized by mutual respect, empathy, and collaboration. 4.b Creates a climate of patient- centered care to include confidentiality, privacy, comfort, emotional support, mutual trust, and respect. 4.c Incorporates the patient's cultural and spiritual preferences, values, and beliefs into health care. duplicate.	Clinical decision making based on evidence and patient/provider partnership Current and emerging professional standards Novice to expert continuum of clinical practice Political, policy and regulatory issues regarding licensure, national certification, and scope of practice. Leadership approaches for employment contract negotiation, networking, and advancing professional standards and roles Application of select sciences to practice: • Pharmacology • Physiology • Pathophysiology Specific areas of assessment, including but not limited to: • Physical • Psychosocial • Developmental • Family • Psychiatric mental health • Oral health Screenings Diagnostics (tests, labs) Specific procedures Health promotion, prevention, and disease management Pharmacology and complementary alternative therapies Provider-patient relationship:

(The National Organization of Nurse Practitioners Faculties [NONPF], 2014, p. 12) (Used with permission)

Table 6d. Competencies Most Applicable to Evaluation and Management of Bleeding Disorders in Women

Independent Practice Competencies	1. Functions as a licensed independent practitioner. 2. Demonstrates the highest level of accountability for professional practice. 3. Practices independently managing previously diagnosed and undiagnosed patients. 3.a Provides the full spectrum of health care services to include health promotion, disease prevention, health protection, anticipatory guidance, counseling, disease management, palliative, and end of life care. 3.b Uses advanced health assessment skills to differentiate between normal, variations of normal and abnormal findings. 3.c Employs screening and diagnostic strategies in the	Provides culturally appropriate reproductive and primary care for women of all ages. Approaches gender-specific developmental events, such as menarche, pregnancy, menopause and senescence, as normative transitions not disease states. Recognizes unique health care needs of marginalized women, including victims of violence and transgendered female clients. Recognizes disease manifestations unique to women. Manages disease manifestations unique to women. Provides infertility and sexually transmitted disease services to sexual partners of female patients. Supports a woman's right to make her own decisions regarding her health and reproductive	Age-appropriate care • women across the lifespan • gynecologic • obstetric Normal vs. abnormal • development of the female • obstetrics • gynecology • age-related changes Male conditions related to reproductive and urologic systems Selection and implementation of appropriate clinical guidelines and standards Using clinical decision support tools Epidemiology/risk analysis, including knowledge of: • Prevalence of gynecologic and obstetric disorders in diverse populations across the

Competency Area	NP Core Competencies	Women's Health / Gender-Related NP Competencies	Curriculum Content to Support Competencies *Neither required nor comprehensive, this list reflects only suggested content specific to the population*
	development of diagnoses. 3.d Prescribes medications within scope of practice. 3.e Manages the health/illness status of patients and families over time. 4. Provides patient-centered care recognizing cultural diversity and the patient or designee as a full partner in decision-making. 4.a Works to establish a relationship with the patient characterized by mutual respect, empathy, and collaboration. 4.b Creates a climate of patient-centered care to include confidentiality, privacy, comfort, emotional support, mutual trust, and respect. 4.c Incorporates the patient's cultural and spiritual preferences, values, and beliefs into health care. 4.d Preserves the patient's control over decision making by negotiating a mutually acceptable plan of care.	choices within the context of her belief system. Assesses genetic, social, environmental, physical, and mental health risks through collection of family, social, environmental, and health data. Provides counseling, management, and/or referral based on identified healthcare risk factors.	life span • Contributing risk factors and potential barriers to health promotion and disease prevention (e.g., socioeconomic, biological, environmental, community-specific variables) Gender-based recommendations, exercise, lifestyle, familial factors that predisposes one to disease, cultural, and societal influences/stigmas. Growth and development theories and concepts (spiritual, cultural, cognitive, emotional, psychosexual, physical abilities) & variances Principles of family dynamics and social support systems. Cultural differences impacting health such as, but not limited to: • language • ethnicity • race • religious • spiritual • biopsychosocial • urban/rural • homeless • migrant • lesbian-gay-bisexual-transgender/transsexual (LGBT) orientation

Table 6d. (Continued)

Competency Area	NP Core Competencies	Women's Health / Gender-Related NP Competencies	Curriculum Content to Support Competencies *Neither required nor comprehensive, this list reflects only suggested content specific to the population*
			• corrections/forensic • uninsured and underinsured • health disparities • health literacy Complementary/alternative medicine therapies used across the lifespan in women's health Crisis management • sexual assault • violence (such as, but not limited to, intimate partner and elder abuse) • divorce • caregiver burden Female genital health, including, but not limited to: • vulvodynia • vulvar vestibulitis • chronic pelvic pain • vulvovaginal dermatalogic conditions Common urological disorders in women, including, not limited to: • urinary Incontinence • urinary frequency • interstitial cystitis Skill in the procedures such as, but not limited to: • IUD insertion • punch biopsies • endometrial biopsies • basic ultrasound

Competency Area	NP Core Competencies	Women's Health / Gender-Related NP Competencies	Curriculum Content to Support Competencies *Neither required nor comprehensive, this list reflects only suggested content specific to the population*
			• pessary use Prenatal and postpartum management, including, but not limited to: • supervision of high-risk pregnancy • breastfeeding • contraception counseling

(The National Organization of Nurse Practitioners Faculties [NONPF], 2013, pp 83-86)

Ob-Gyn Resident Survey

In 2009, the authors sent a 24-item questionnaire to ob-gyn chief residents at 241 non-military ob-gyn residency programs, and results were published in 2011 (Dietrich, Tran and Giardino, 2011). (See Appendix I for questionnaire.) After exclusions, an overall response rate of 30% (71/239) was achieved. Residents reported training in the medical evaluation of menorrhagia during residency with a mean of 9.1 hours per year in the first year of residency and 11.1 hours/year in the second, third, and fourth years; 67.7% reported that they viewed their training in the medical evaluation of menorrhagia and bleeding disorders as sufficient preparation for clinical practice; and over two thirds reported specific training in common bleeding disorders, such as VWD.

The questionnaire contained clinical scenarios, and the residents reported they would be more likely to perform a bleeding evaluation for menorrhagia at menarche (84.6%) rather than postpartum hemorrhage or menorrhagia as an adult woman post-childbearing. The residents also reported specific training on VWD type 1 (78.5%), VWD type 2 (67.7%), and VWD type 3 (63.1%). Table 7 a-d contains additional data:

Tables 7. Ob-Gyn Chief Resident Survey Results

Table 7a. (p.4)

Program Teaching Format on the Evaluation of Menorrhagia

Teaching format	% of resident respondents who reported teaching in this format N = 65
Occasional didactic lectures on dysfunctional uterine bleeding	96.9
Addressed bleeding disorders	83.1
Systematically taught throughout training	66.2
Dysfunctional uterine bleeding in REI lectures	63.1
Clinical care/evaluation with faculty supervision	90.8
Clinical care/evaluation without faculty supervision	78.5
Pediatric gynecology lectures (if program present)	43
General gynecology lectures	90.8

Table 7b. (p.3)

Risk Situations

Disorders that may have been included in training sessions during residency	Situations they believe increase the risk for a bleeding disorder	Situations that may make a bleeding disorder better or worse
VWD Type 1	Family history	Hormones
VWD Type 2	Menorrhagia at menarche	Pregnancy
VWD Type 3	Menorrhagia any time	Combined hormonal contraceptives
Bernard-Soulier syndrome	Easy bruising	Blood type
ITP	Gum bleeds	Thyroid disease
TTP	Hematomas	Exercise
Anemia of chronic disease	Nosebleeds	Stress
Iron deficiency anemia	Blood type	Liver/renal disease
Blackfan-Diamond syndrome	Thyroid disease	Aspirin
Hemophilia	Other chronic conditions	NSAIDs
Glanzmann thrombasthenia		Tylenol

Table 7c. (p.3)

Percent of Chief Residents Reporting on Patient Menstrual Bleeding History

Frequency	Cycle length	Days of bleeding	Number of Pads/ Tampons used within 24 hours	Post partum hemorrhage in the past
Always	61.5	58.5	23.1	9.2
Most of the time	26.5	30.8	41.5	13.8
Usually	9.2	7.7	16.9	26.2
Sometimes	1.5	1.5	12.3	26.2
Less than half of the time	0	0	4.6	20
Never	0	0	0	3.1

N = 65; values are percentage

Table 7d. (p.4)

Percent of Chief Residents Reporting on Patient Non-Menstrual Bleeding History

Frequency	Nosebleeds N = 54	Gum bleeding N = 63	Thyroid abnormalities N = 63	Easy Bruising N = 63	Upon review of Surgical history, excessive bleeding after T&A N = 64
Always	1.5	1.5	16.9	9.2	6.2
Most of the time	3.1	3.1	38.5	16.9	6.2
Usually	4.6	9.2	15.4	18.5	6.2
Sometimes	20	23.1	21.5	15.4	13.8
Less than half of the time	46.2	43.1	4.6	29.2	29.2
Never	21.5	16.9	0	7.7	36.9

(Dietrich, Tran and Giardino, 2011) (Used with permission)

Ob-gyn chief residents self-reported that they do receive training via didactics and clinical precepting in the evaluation of dysfunctional uterine bleeding and perceive their training to be sufficient for clinical practice. While

the majority of respondents included appropriate screening questions during history taking in the face of a potential bleeding disorder, fewer residents were aware of risk factors and clinical scenarios suggestive of bleeding disorders. Although most residents knew of situations that could improve or worsen bleeding conditions in the clinical setting, a sizeable number did not perform as well in the clinical management of dysfunctional uterine bleeding. The CREOG Educational Objectives (2013; 2016) call for such training. It is rewarding to see that the responding programs provided an average of 9.1 hours of training in the first year and an average of 11.1 hours in subsequent years of training. Still, there is room for improvement.

The NHLBI (2007) VWD guidelines place an emphasis on the medical history component of the bleeding disorder evaluation, and the residents who responded to this survey demonstrated an appropriate history. In response to clinical scenarios, a number of chief residents demonstrated an understanding of the basic laboratory and imaging workup, although there was limited understanding specifically for the evaluation of bleeding disorders. The survey also revealed that 76% of the ob-gyn chief residents thought their training was sufficient for the evaluation of bleeding disorders. (See Table 7 a-d.)

Women's Health Advanced Practice Nurses

In 2012, all 2224 APNs who were members of the National Association of Nurse Practitioners in Women's Health were sent an email containing a 25-question anonymous survey assessing training experiences and knowledge in the evaluation of heavy menstrual bleeding and bleeding disorders in women (see Appendix II for questionnaire). After exclusions, a response rate after two rounds was 17% (N = 370). Mean age of participants was 49.8 years. Graduation years from a nurse practitioner program ranged from 1978 to 2012. Three-fourths (75.1%) of the participants identified themselves as Women's Health NPs, and 90% of the participants were currently treating patients with heavy menstrual bleeding. The study will be published in 2017 (Kurkowski, Giardino, Sangi and Dietrich, 2017). Table 8 contains additional data.

Bleeding disorders were addressed in 76% of the participants' nurse practitioner training programs. Sixty-two percent of participants viewed their training as insufficient in the medical evaluation of bleeding disorders in

preparation for clinical practice. Nearly 16% reported that heavy menstrual bleeding at menarche would prompt a bleeding disorder workup. Over 43% of participants reported that heavy menstrual bleeding as an adult woman or postpartum hemorrhage would prompt them to consider a bleeding disorder workup. Over 65% reported they would order, as part of the clinical workup, no more than a complete blood count and thyroid-stimulating hormone for a patient with a history of heavy menstrual bleeding. When asked about education in school for VWD specifically, over half (59.5%) reported learning about type 1 VWD and less than 16% reported learning about types 2 and 3.

The NPs whose programs addressed bleeding disorders were more likely to ask more questions about bleeding history, such as number of pads and tampons changed in a 24-hour period. VWD surprisingly was not reported as the most commonly taught bleeding disorder. Structured training in women's health that addresses the basics of bleeding disorders should be included in the Women's Health curriculum as advocated by HRSA (2012) in the section above.

Table 8. Additional Data from 2012 APNs Survey

Training Received by Study Participants		
For the training you received in the evaluation of menorrhagia, did you receive teaching in any of the following formats?		
Answer Options	Yes	No
A. Occasional didactic presentations on Dysfunctional Uterine Bleeding?	89.91%	10.09%
B. Addressed bleeding disorders?	76.39%	23.60%
C. Systematically taught throughout training?	53.45%	47.16%
D. Clinical care/evaluation, supervised by preceptor?	74.30%	25.69%
E. Clinical care/evaluation, without preceptor supervision?	45.63%	54.69%
F. Taught in Pediatric and Adolescent Gynecology	30.58%	70.44%
G. Taught in General Gynecology	87.26%	12.73%

Table 8. (Continued)

Bleeding Disorder clinical work-up			
Training Sufficient in Bleeding Disorders	Yes 37.38% N = 140	No/No opinion 62.16% N = 230	P value
Routinely sent lab tests	124 (88.6%)	164 (71.3%)	.0001
CBC	24 (17.1%)	15 (6.5%)	.001
Factor VIII	34 (24.3%)	21 (9.1%)	<.0001
VW panel	111 (79.3%)	136 (59.1%)	<.0001
TSH	36 (25.7%)	37 (16.1%)	.02
Coags	1 (.7%)	1 (.4%)	.99
VW multimer	1 (.7%)	2 (.9%)	.99
Ristocetin cofactor	0 (0%)	2 (.9%)	.53
TEG	53 (37.9%)	63 (27.4%)	.04
Iron panel	14 (10%)	23 (10%)	.99
Fibrinogen	1 (.7%)	4 (1.7%)	.41
PFA-100	0 (0%)	0 (0%)	---
All	5 (3.6%)	12 (5.2%)	.46
None			

Training Received, by Specific Disorder

Please check the following disorders which may have been included in a training session on evaluation of menorrhagia in your advanced practice nursing program. (Check all that apply)

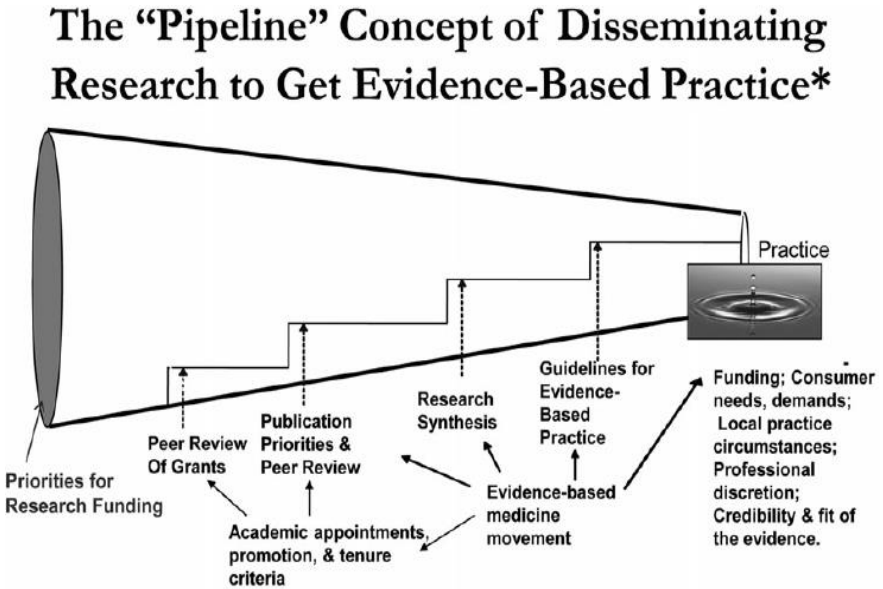
Answer Options	Response Percent
Von Willebrand disease type 1	59.5%
Von Willebrand disease type 2	15.7%
Von Willebrand disease type 3	14.6%
Bernard–Soulier syndrome	0.8%
ITP (idiopathic thromocytopenic purpura)	50.0%
TTP (thrombotic thrombocytopenic purpura)	25.1%
Anemia chronic disease	56.8%
IDA (iron deficiency anemia)	64.9%
Blackfan-Diamond syndrome	0.8%
Hemophilia	45.4%
Glanzman’s thrombasthenia	0.0%

CONCLUSION/DISCUSSION

The promulgation of the NHLBI VWD guidelines provides an excellent framework from which to improve the current training and move toward "best practices." However, the adoption of best practices takes time in health care, as demonstrated by Green's (2008) summary of scholarly work where he describes a pipeline of sorts and suggests, owing to rigors of peer review and the typical academic process, that it may take many years for evidence to amass and for a new standard of care to be adopted. Figures X.5 and X.6 highlight the many steps along the peer-reviewed academic process for an idea to be tested and refined and come to be seen as evidence.

Experience in many field bears witness to this arduous process. In pediatrics, for example, Cabana and colleagues (2000) have extensively studied guideline adoption around the care of asthmatic patients. Examining the barriers identified to adopting a set of NHLBI evidence-based guidelines on asthma care that were widely disseminated, Cabana and colleagues (2000) found that the providers themselves may not be aware, familiar or agree with the guideline. Additional practice logistics and providers' perceptions of applicability to one's patient population can also be impediments to adoption of the guideline, as are other factors such as lack of confidence that they will have an impact as well as what was termed practice inertia (takes time to make changes in already established).

The 2007 NHLBI VWD guidelines provide an excellent example of the academic peer-review process identified by Green that leads to the promulgation of evidence meant to impact practice. Fortunately, training in the area of bleeding disorder evaluation and VWD evaluation and management for ob-gyn residents and Women's Health NPs appears to be in place, but there is room for improvement. As would be expected, the training appears to be a mix of didactic and clinical precepting at the bedside, which seems appropriate. The amount of time dedicated to both didactic and clinical training is reasonable in view of the many competing time demands in the professional development curriculum. By self-report, ob-gyn residents think they are being adequately prepared for clinical practice, and Women's Health NPs, while receiving training in bleeding disorders, would like to see more training to prepare them for practice.



*Based on Green L.W. from research to "best practice" in other setting and populations. Am. J. Health Behavior 25: 165-178, April-May 2001.

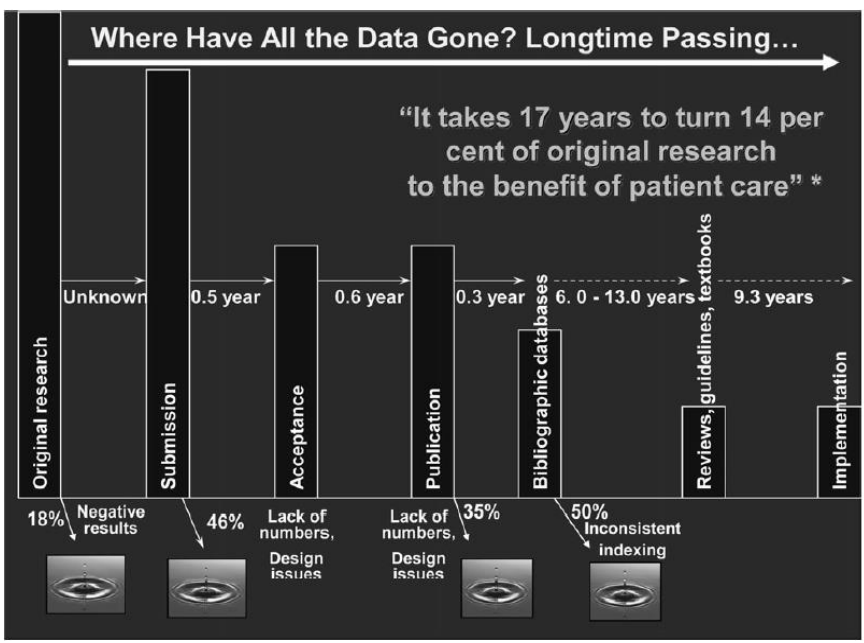
Green, L. (2008). Making research relevant: If it is an evidence-based practice, where's the practice based evidence? Family Practice, 25, 120-124. (Used with permission)

"The pipeline conceptualization and implementation of transferring to practice results in successive constrictions of the flow of knowledge and an 'evidence-based guideline' product at the practitioner end of the pipeline that has a poor fit with practice circumstances such as funding, time constraints and patient demands" (Green, 2008).

Figure 5. Green's Evidence Pipeline and Timeline

The value of the initial history is recognized by both groups of providers, and this supports the emphasis placed by the NHLBI guidelines on the screening and diagnostic value of the initial medical history taking. Considering the responses to clinical scenarios, ob-gyn residents demonstrate a sound grasp of the basic laboratory and imaging workup essential to the VWD guidelines, as do the Women's Health NPs. The 2007 NHLBI VWD guidelines are an excellent practice aid that when adopted and adhered to can help address common bleeding disorders that will be seen by providers who

serve female patients across the life span. Realizing that self-report on a survey questionnaire may not reflect actual practice, and recognizing that many factors beyond knowledge of the existence of a guideline may impact adoption and adherence to it, we recommend that continued education be incorporated in the professional training of physicians and NPs with an emphasis on history-taking questions and physical examination findings that can serve as a guide to what laboratory and imaging studies may be required for accurate diagnosis and subsequent management decision making.



The leakage points in the flow of original research into practice and the lag time between points as estimated by Balas from a variety of sources. Source: based on data reviewed and summarized by Balas, E.A. and Boren, S.A. (2000). Managing clinical knowledge for health care improvement. Yearbook of Medical Informatics 2000: Patient-centered Systems. Stuttgart, Germany: Schattauer,65-70. Green, L. (2008). Making research relevant: If it is an evidence-based practice, where's the practice based evidence? Family Practice, 25, 120-124. (Used with permission)

Figure 6. Flow of Original Research into Practice

APPENDIX I: BLEEDING DISORDER EVALUATION CHIEF RESIDENT QUESTIONNAIRE

I. Demographics

1. Your Gender (Please check one) Male _____ Female _____

2. Size of residency program of which you are the Chief Resident (Please fill in)
Total #_____: PGY-4 ___ PGY-3 ___ PGY-2 ___ PGY-1 ___

3. Setting that best describes where most of the patients served by your program reside: (Please check one): ____ Urban ___ Suburban ___ Rural ___ Not Sure

4. The patient population your residency program serves is what percent of each of the following? Private _____ Public _____

5. Approximately how many hours of teaching in the medical evaluation of menorrhagia did you receive in each year of training?

a) PGY-1 ____ hrs/yr __ not sure
b) PGY-2 ____ hrs/yr __ not sure
c) PGY-3 ____ hrs/yr __ not sure
d) PGY-4 ____ hrs/yr __ not sure

6. For the training you received in the evaluation of menorrhagia, did you receive the teaching in any of the following formats?

	YES	NO
a) Occasional didactic presentations on Dysfunctional Uterine Bleeding?	____	____
b) Addressed bleeding disorders?	____	____
c) Systematically taught throughout training?	____	____
d) Dysfunctional Uterine Bleeding - Taught in REI?	____	____
e) Clinical care/evaluation, supervised by faculty?	____	____
f) Clinical care/evaluation, without faculty supervision?	____	____
g) Other training that you received:		
1. Taught in Pediatric and Adolescent Gynecology?	____	____

2. Taught in General Gynecology ____ ____

7. Do you consider the training in the medical evaluation of bleeding disorders to have been sufficient preparation for your clinical practice?
___ Yes___ No___ No Opinion

8. During your four years of residency training approximately how many bleeding disorders evaluations did you perform? (Please check one)
None ___ 1-10 ___ 1-20 ___ 21-30 ___ 31-40 ___ 41-50 ___ > 50 ___

9. As a Chief Resident, have you performed any bleeding disorder workups for menorrhagia this year? (Please check one)
Yes ___ If yes, how many? ___ No ___ Not sure ___

10. During your 4 years of residency, how often do you ask about cycle length with regard to Gynecology history?

a) Always (100% of the time)
b) Most of the time (>90%)
c) Usually (70-90%)
d) Sometimes (50-69%)
e) Less than half of the time (1-49%)
f) Never (0%)

11. During your 4 years of residency, how often do you ask about days of bleeding with regard to Gynecology history?

a) Always (100% of the time)
b) Most of the time (>90%)
c) Usually (70-90%)
d) Sometimes (50-69%)
e) Less than half of the time (1-49%)
f) Never (0%)

12. During your 4 years of residency, how often do you ask a menstruating patient about the number of pads and/or tampons used within a 24-hour• period?

a) Always (100% of the time)

b) Most of the time (>90%)
c) Usually (70-90%)
d) Sometimes (50-69%)
e) Less than half of the time (1-49%)
f) Never (0%)

13. During your 4 years of residency, how often do you ask about postpartum hemorrhage?

a) Always (100% of the time)
b) Most of the time (>90%)
c) Usually (70-90%)
d) Sometimes (50-69%)
e) Less than half of the time (1-49%)
f) Never (0%)

II. Practice

1. How often do you routinely ask about frequent nosebleeds?

a) Always (100% of the time)
b) Most of the time (>90%)
c) Usually (70-90%)
d) Sometimes (50-69%)
e) Less than half of the time (1-49%)
f) Never (0%)

2. How often do you ask about gum bleeding?

a) Always (100% of the time)
b) Most of the time (>90%)
c) Usually (70-90%)
d) Sometimes (50-69%)
e) Less than half of the time (1-49%)
f) Never (0%)

3. How often do you ask about thyroid abnormalities?

a) Always (100% of the time)
b) Most of the time (>90%)
c) Usually (70-90%)
d) Sometimes (50-69%)
e) Less than half of the time (1-49%)
f) Never (0%)

4. How often do you ask about easy bruising?

a) Always (100% of the time)
b) Most of the time (>90%)
c) Usually (70-90%)
d) Sometimes (50-69%)
e) Less than half of the time (1-49%)
f) Never (0%)

5. Upon review of a patient's surgical history, how often do you inquire about excessive bleeding following a tonsillectomy and adenoidectomy?

a) Always (100% of the time)
b) Most of the time (>90%)
c) Usually (70-90%)
d) Sometimes (50-69%)
e) Less than half of the time (1-49%)
f) Never (0%)

6. How often do you routinely order an ultrasound with an adolescent with menorrhagia?

a) Always (100% of the time)
b) Most of the time (>90%)
c) Usually (70-90%)
d) Sometimes (50-69%)
e) Less than half of the time (1-49%)
f) Never (0%)

7. Given a history of heavy periods, which of the following would you send routinely? (Please check all that apply)

____ a. CBC
____ b. Factor VIII
____ c. Von Willebrand factor
____ d. TSH
____ e. PT, PTT, INR
____ f. PFA-100
____ g. Von Willebrand multimers
____ h. Ristocetin cofactor
____ i. TEG
____ j. All of the above
____ k. None of the above

8. For the following clinical scenarios, what would prompt you to perform a bleeding disorder workup?

a) Menorrhagia at menarche
b) Post partum hemorrhage
c) Menorrhagia as an adult woman post-child bearing

III. Risk Situations

1. Please check the following disorders which may have been included in a training session on evaluation of menorrhagia (check all that apply).

_____ VWD Type 1
_____ VWD Type 2
_____ VWD Type 3
_____ Bernard-Soulier syndrome
_____ ITP
_____ TTP
_____ Anemia chronic disease
_____ IDA
_____ Blackfan-Diamond syndrome
_____ Hemophilia
_____ Glanzman’s thrombasthenia

2. Please check those situations which you believe increase the risk for bleeding disorders. (Check all that apply)

_____ Family history
_____ Menorrhagia only at menarche
_____ Menorrhagia anytime
_____ Easy bruising
_____ Gum bleeds
_____ Hematoma development
_____ Nosebleeds
_____ Blood type
_____ Thyroid disease
_____ Other chronic conditions such as renal or liver disease

3. Please mark which situations may make a bleeding condition better or worse. (Check better, worse or not applicable for letters a-k)

	Better	Worse	Not Applicable
a) Hormone	_____	_____	_____
b) Pregnancy	_____	_____	_____
c) CCPS	_____	_____	_____
d) Blood type	_____	_____	_____
e) Thyroid disease	_____	_____	_____
f) Exercise	_____	_____	_____
g) Stress	_____	_____	_____
h) Liver/renal disease	_____	_____	_____
i) Aspirin	_____	_____	_____
j) NSAID	_____	_____	_____
k) Tylenol	_____	_____	_____

Please check her if you would like a copy of "A Pocket Guide to the Diagnosis, Evaluation and Management of Von Willebrand Disease."

Thank you for your participation in our survey!

APPENDIX II: BLEEDING DISORDER EVALUATION NURSE PRACTITIONER QUESTIONNAIRE

I. Demographics

1. Your Gender? *(Please check one)* Male _____ Female _____

2. What is your age? ____

3. What level of education prepared you for your current role as an advanced practice nurse?

Bachelor's Degree _____ Master's Degree ___ Doctoral Degree ___ Other ___

4. What year did you graduate from your advanced practice nurse program? _____

5. Which type of advanced practice nurse are you? *(Please check one)*
WHNP _____ FNP ___ ANP ___ CNM ___ Other___

6. Do you treat patients with menorrhagia? Yes_____ No ___

7. Setting that best describes where most of the patients served by your advanced practice nurse program reside: *(Please check one)* ____ Urban ___ Suburban ___ Rural ___ Not Sure

II. Clinical Experience during Your Advanced Practice Nursing Program

1. For the training you received in the evaluation of menorrhagia, did you receive teaching in any of the following formats?

	YES	NO
a) Occasional didactic presentations on Dysfunctional Uterine Bleeding?	____	____
b) Addressed bleeding disorders?	____	____
c) Systematically taught throughout training?	____	____

d) Clinical care/evaluation, supervised by preceptor? ____ ____
e) Clinical care/evaluation, without preceptor supervision? ____ ____
f) Other training that you received:
   1. Taught in Pediatric and Adolescent Gynecology? ____ ____
   2. Taught in General Gynecology ____ ____

2. Do you consider the training you recieved in the medical evaluation of bleeding disorders to have been sufficient preparation for your clinical practice? *(Please check one)*

___ Yes___ No ___ No Opinion

3. During your advanced practice nurse training approximately how many bleeding disorders evaluations did you perform? *(Please check one)*

None ___ 1-10 ___ 1-20 ___ 21-30 ___ 31-40 ___ 41-50 ___ >than 50 ___

4. During your advanced practice nursing program, how often do you ask about cycle length with regard to Gynecology history? *(Please check one)*

a) Always (100% of the time)
b) Most of the time (>90%)
c) Usually (70-90%)
d) Sometimes (50-69%)
e) Less than half of the time (1-49%)
f) Never (0%)

5. During your advanced practice nursing program, how often do you ask about days of bleeding with regard to Gynecology history? *(Please check one)*

a) Always (100% of the time)
b) Most of the time (>90%)
c) Usually (70-90%)
d) Sometimes (50-69%)
e) Less than half of the time (1-49%)
f) Never (0%)

6. During your advanced practice nursing program, how often do you ask a menstruating patient about the number of pads and/or tampons used within a 24-hour period? *(Please check one)*

a) Always (100% of the time)
b) Most of the time (>90%)
c) Usually (70-90%)
d) Sometimes (50-69%)
e) Less than half of the time (1-49%)
f) Never (0%)

7. During your advanced practice nursing program, how often do you ask about post partum hemorrhage? *(Please check one)*

a) Always (100% of the time)
b) Most of the time (>90%)
c) Usually (70-90%)
d) Sometimes (50-69%)
e) Less than half of the time (1-49%)
f) Never (0%)

8. Please check the following disorders which may have been included in a training session on evaluation of menorrhagia in your advanced practice nursing program *(Check all that apply).*

_____ Von Willebrand disease type 1
_____ Von Willebrand disease type 2
_____ Von Willebrand disease type 3
_____ Bernard-Soulier syndrome
_____ ITP (idiopathic thromocytopenic purpura)
_____ TTP (thrombotic thrombocytopenic purpura
_____ Anemia chronic disease
_____ IDA (iron deficiency anemia)
_____ Blackfan-Diamond syndrome
_____ Hemophilia
_____ Glanzman's thrombasthenia

III. Current Practice

1. As an Advanced Practice Nurse, have you performed any bleeding disorder workups for menorrhagia this year? *(Please check one)*

Yes ___ If yes, how many? ___ No___ Not sure

2. How often do you routinely ask about frequent nosebleeds? *(Please check one)*

a) Always (100% of the time)
b) Most of the time (>90%)
c) Usually (70-90%)
d) Sometimes (50-69%)
e) Less than half of the time (1-49%)
f) Never (0%)

3. How often do you ask about gum bleeding? *(Please check one)*

a) Always (100% of the time)
b) Most of the time (>90%)
c) Usually (70-90%)
d) Sometimes (50-69%)
e) Less than half of the time (1-49%)
f) Never (0%)

4. How often do you ask about thyroid abnormalities? *(Please check one)*

a) Always (100% of the time)
b) Most of the time (>90%)
c) Usually (70-90%)
d) Sometimes (50-69%)
e) Less than half of the time (1-49%)
f) Never (0%)

5. How often do you ask about easy bruising? *(Please check one)*

a) Always (100% of the time)
b) Most of the time (>90%)
c) Usually (70-90%)
d) Sometimes (50-69%)
e) Less than half of the time (1-49%)
f) Never (0%)

6. Upon review of a patient's surgical history, how often do you inquire about excessive bleeding following a tonsillectomy and adenoidectomy? *(Please check one)*

a) Always (100% of the time)
b) Most of the time (>90%)
c) Usually (70-90%)
d) Sometimes (50-69%)
e) Less than half of the time (1-49%)
f) Never (0%)

7. How often do you routinely order an ultrasound with an adolescent with menorrhagia? *(Please check one)*

a) Always (100% of the time)
b) Most of the time (>90%)
c) Usually (70-90%)
d) Sometimes (50-69%)
e) Less than half of the time (1-49%)
f) Never (0%)

8. Given a history of heavy periods, which of the following would you send routinely? (*Please check all that apply)*

____ a. CBC
____ b. Factor VIII
____ c. Von Willebrand factor
____ d. TSH (thyroid-stimulating hormone)
____ e. PT, PTT, INR coagulation panel
____ f. PFA-100 platelet function analyzer 100
____ g. Von Willebrand multimers
____ h. Ristocetin cofactor
____ i. TEG (thromboelastogram)
____ j. Iron panel
____ k. Fibrinogen
____ l. All of the above
____ m. None of the above

9. For the following clinical scenarios, what would prompt you to perform a bleeding disorder workup? *(Check all thay apply)*

a) Menorrhagia at menarche
b) Postpartum hemorrhage
c) Menorrhagia as an adult woman post-child bearing

IV. Risk Situations

1. Please check those situations which you believe increase the risk for bleeding disorders. (*Check all that apply*)

_____ Family history
_____ Menorrhagia only at menarche
_____ Menorrhagia anytime
_____ Easy bruising
_____ Gum bleeds
_____ Hematoma development
_____ Nosebleeds
_____ Blood type
_____ Thyroid disease
_____ Other chronic conditions such as renal or liver disease

2. Please mark which situations may make a bleeding condition better or worse? *(Check better, worse or not applicable for letters a-k)*

	Better	Worse	NotApplicable
a) Hormone	_____	_____	_____
b) Pregnancy	_____	_____	_____
c) Oral contraceptive pills	_____	_____	_____
d) Blood type	_____	_____	_____
e) Thyroid disease	_____	_____	_____
f) Exercise	_____	_____	_____
g) Stress	_____	_____	_____
h) Liver/renal disease	_____	_____	_____
i) Aspirin	_____	_____	_____
j) NSAIDs	_____	_____	_____
k) Tylenol	_____	_____	_____

Please check her if you would like a copy of "A Pocket Guide to the Diagnosis, Evaluation and Management of Von Willebrand Disease" (yes or no). Participant needs to leave name and address.

Thank you for your participation in our survey!

REFERENCES

American Association of Colleges of Nursing. (2002). Nurse Practitioner Primary Care Competencies in Specialty Areas: Adult, Family, Gerontological, Pediatric, and Women's Health.

American Association of Colleges of Nursing. (2016) Adult-Gerontology Acute Care and Primary Care NP Competencies. http://www.aacn.nche.edu/education-resources/Adult-Gero-NP-Comp-2016.pdf.

American College of Obstetricians and Gynecologist (AGOG). (2009). Council on Resident Education in Obstetrics and Gynecology (CREOG): Units 4 and 5. CREOG Objectives, (9th ed.). ACOG. Washington DC, Professional Publishing Group.

American College of Obstetricians and Gynecologists (ACOG). (2013). Educational Objectives: Core Curriculum in Obstetrics and Gynecology, 10th Edition. Council on Resident Education in Obstetrics and Gynecology. http://med.unr.edu/Documents/unsom/obgyn/CREOGEducationalObjectives10thEdition.pdf.

American College of Obstetricians and Gynecologists (AGOC). (2015). Committee Opinion. Committee on Adolescent Health Care and Committee on Gynecologic Practice. http://www.acog.org/Resources-And-Publications/Committee-Opinions/Committee-on-Adolescent-Health-Care/Von-Willebrand-Disease-in-Women

American College of Obstetricians and Gynecologists (AGOG). (2016). *Educational Objectives: Core Curriculum in Obstetrics and Gynecology*, 11th Edition. Council on Resident Education in Obstetrics and Gynecology. http://www.acog.org/About-ACOG/ACOG-Departments/CREOG/CREOG-Search/CREOG-11th-Edition-of-Educational-Objectives.

Balas, E. A. and Boren, S.A. (2000). Managing clinical knowledge for health care improvement. *Yearbook of Medical Informatics 2000: Patient-centered Systems*. Stuttgart, Germany: Schattauer. pp. 65-70.

Cabana, M., Ebel, B.E., Cooper-Patrick, L, Powe, N.R., Rubin, H.R., and Rand, C.S. (2000). Barriers Pediatricians Face When Using Asthma

Practice Guidelines. *Archives of Pediatric Adolescent Medicine*, 154, 685-693.

Dietrich, J.E., Tran, X.G. and Giardino, A.P. (2011). Bleeding disorder education in obstetrics and gynecology residency training: a national survey. *Journal of Pediatric and Adolescent Gynecology*, 24, 94-97.

Green, L. (2008). Making research relevant: If it is an evidence-based practice, where's the practice based evidence? *Family Practice*, 25, 120-124.

Healthy People 2020. (2016). About Healthy People. Washington, DC: US Department of Health and Human Services, Office of Disease Prevention and Health Promotion. https://www.healthypeople.gov/2020/About-Healthy-People.

Healthy People 2020. (2016). Blood Disorders and Blood Safety. BDBS-14 https://www.healthypeople.gov/node/4026/data_details#revision_history_header.

Healthy People 2020. (2016). Blood Disorders and Blood Safety. BDBS-15 https://www.healthypeople.gov/node/4027/data_details#revision_history_header.

Kurkowski, J.P., Giardino, A.P., Sangi, H.H., Dietrich, J.E. (2017). Brief Report: Von Willebrand Disease in Women: A Review and APN Survey Results. International Archives of Nursing and Health Care.

National Heart, Lung, and Blood Institute (NHLBI). (2004). Women with Bleeding Disorders. https://www.nhlbi.nih.gov/research/reports/2004-women-bleeding-disorders.

National Heart, Lung, and Blood Institute (NHLBI). (2007). *Full Report: The Diagnosis, Evaluation, and Management of von Willebrand Disease*. Bethesda, MD: US Department of Health and Human Services, National Institutes of Health; NIH publication 08-5832. https://www.nhlbi.nih.gov/files/docs/guidelines/vwd.pdf.

National Heart, Lung, and Blood Institute (NHLBI). (2011). What Is Von Willebrand Disease? https://www.nhlbi.nih.gov/health/health-topics/topics/vwd.

National Heart, Lung, and Blood Institute (NHLBI). (2013). What Is Hemophilia? https://www.nhlbi.nih.gov/health/health-topics/topics/hemophilia.

National Organization of Nurse Practitioners Faculties. (2012). Nurse Practitioners Core Competencies. http://c.ymcdn.com/sites/www.nonpf.org/resource/resmgr/competencies/npcorecompetenciesfinal2012.pdf.

National Organization of Nurse Practitioners Faculties. (2013). Population-Focused Nurse Practitioner Competencies. http://www.aacn.nche.edu/education-resources/PopulationFocusNPComps2013.pdf.

National Organization of Nurse Practitioners Faculties (NONPF). (2014). Nurse Practitioners Core Competencies Content. http://c.ymcdn.com/sites/nonpf.site-ym.com/resource/resmgr/Competencies/NPCoreCompsContentFinalNov20.pdf.

Population-Focused Competencies Task Force. (2013). Population-Focused Nurse Practitioner Competencies: Family/Across the Lifespan, Neonatal, Pediatric Acute Care, Pediatric Primary Care, Psychiatric-Mental Health, Women's Health/Gender-Related. http://c.ymcdn.com/sites/www.nonpf.org/resource/resmgr/Competencies/CompilationPopFocusComps2013.pdf.

Sadler, J.E., Budde, U., Eikenboom, J.C.J., Favaloro, E.J., Hill, F.G.H. et al. (2006). Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand Factor. *Journal of Thrombosis and Haemostasis*, 4, 2103-2114.

Seltzer, V.L., Fishburne, J.L. Jr. and Jonas, H.S. (1998). Obstetrics and gynecology residencies: education in preventive and primary health care for women. *Obstetrics and Gynecology*, 9,305.

Teunissen, P.W., Stapel, D.A., Scheele, F., et al. (2009). The influence of context on resident's evaluations: effects of priming on clinical judgment and affect. *Advances in Health Science Education: Theory and Practice*, 14,23.

US Department of Health and Human Services. Health Resources and Services Administration. (2002). Nurse Practitioner Primary Care competencies in Specialty Area: Adult, Family Gerontological, Pediatric, and Women's Health. http://www.aacn.nche.edu/education-resources/npcompetencies.pdf.

US Department of Health and Human Services, Health Resources and Services Administration, Office of Women's Health. Women's health curricula: Final report on expert panel recommendations for interprofessional collaborations across the Health Professions. Rockville, Maryland: US Department of Health and Human Services, 2013.

Weiss J. (2012). Just heavy menses or something more? Raising awareness of von Willebrand disease. *American Journal of Nursing*, 12(6), 38-44.

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Chapter 9

MICROBIAL DYNAMICS CHARACTERIZATION DURING THE COMPOSTING PROCESS OF ORGANIC WASTES

Loubna El Fels[1,2,*], Abdelghani El Asli[3], Souad Loqman[4], Yedir Ouhdouch[5] and Mohamed Hafidi[2]

[1]Higher Institute of Nursing Professions and Health Technics, Marrakech-Safi, Morocco
[2]Laboratory of Ecology and Environment (L2E) (Unit Associated with the CNRST, URAC32), Faculty of Science Semlalia, Cadi Ayyad University, Marrakech, Morocco
[3]School of Science and Engineering, Al Akhawayn University of Ifrane, Ifrane, Morocco
[4]Laboratory of Microbiology-Virology Faculty of Medicine and Pharmacy, Cadi Ayyad University, Morocco
[5]Laboratory of Biology and Biotechnology of Microorganisms, Faculty of Science Semlalia, Cadi Ayyad University, Marrakech, Morocco

[*] Corresponding author: Pr Loubna EL FELS, E-mail: loubna.elfels@gmail.com

ABSTRACT

Composting process is a natural pathway to convert organic matter into humic substances end product. The objective of this study is focused mainly on the characterization of the microflora, which plays a key role as decomposers during the composting process and its interaction with physico-chemical parameters. Mesophilic microflora constitutes the pioneer component, while thermophilic microflora contributes significantly to the quality of the compost especially during the thermophilic stage. Indigenous microflora community changes during composting of activated sewage sludge and date palm waste, and food waste and garden clippings. The thermophilic microflora evolution during composting is linked to ascending temperature (over than 50°C). The mesophilic microflora is found to be more abundant than the thermophilic bacteria throughout the co-composting process. Total microflora followed the temperature profile variation. The values of pH, T°C and C/N ratio are typical values for composting process. The temperature rise and the abatement of C/N ratio during composting of sewage sludge/ date palm and household/green waste indicate the intense microbial activity. Thereafter, the decrease in biomass and temperature show the end of the composting process. Fungal microflora increases during the thermophilic stage with a high correlation rate in sewage sludge-palm waste composting. The changes in microflora explain the organic matter evolution during composting. The dynamics of microbial community in different compost ecosystems varies with qualitative and quantitative changes in physico-chemical conditions of compost, which is linked to the quality of organic matter degradation.

Keywords: composting, microbial diversity, mesophilic and thermophilic microflora, physico-chemical parameters, maturity

1. INTRODUCTION

The decomposition of organic matter by biological processes is a natural path of recycling, and composting is one of the biological tools for decomposing biodegradable organic waste, especially sewage sludge and household organic waste. Solid organic waste composts used as organic fertilizers have beneficial effects on plant growth and are considered as a valuable soil amendment (Gharib et al., 2008). This practice is both agronomically interesting and waste management strategy attracting.

The composting process is characterized by two principal phases, which are stabilization and maturation stage. However, the microorganisms that populate substrates during composting reflect the evolution and the performance of the process. The microbial metabolic pathway influences the physico-chemical parameters, which in turn lead to changes of microbial community succession.

With the required vigilance in monitoring of compost quality prior to land application of solid organic waste and sewage sludge, we expect a sharp variation in reported indigenous microflora. Current composting practice relies on indigenous microorganisms to complete the needed biochemical transformations to achieve a finished, stable product. Stability as defined by low CO_2 respiration and lack of continued self- heating is required.

Numerous microorganisms have been shown to be associated with composted raw matter (Ryckeboer et al., 2003b). It is evident that the microbial community, as a whole, plays an important role in the decomposition of organic materials and in the build-up of stabilized compounds. For most microbial species, however, the precise role in the composting process in unknown. It is important to mention that the presence of a large variety of mesophilic, thermotolerant and thermophilic aerobic microorganisms, including bacteria, actinomycetes, yeasts and various other fungi, have been extensively reported in composts and other self-heating organic materials (Amner et al., 1988; Faure and Deschamps, 1991; Finstein and Morris, 1975; Strom, 1985; Beffa et al., 1996). Many factors determine the microbial community during composting. Indeed, under aerobic conditions, temperature is the major factor that determines the types of microorganisms, species diversity, and the rate of metabolic activities.

Until now, the identity of bacterial species involved in the composting of wastes has not been well studied, probably because of the lack of reliable technics. Current knowledge about the compost microbial community is based on different approaches such as the development of DNA-probe methods (Curiale, 1990) and phenotypic techniques (Krieger, 1992) that represent tools to identify at the genus level and at the species level the bacteria important for composting various wastes.

The direct analysis of phospholipid fatty acid patterns (PLFA) (Klammer and Baath, 1998) or molecular techniques (Riddech et al., 2002) are also used. Klammer et al. (2008) differentiated microbial communities of mature compost originating from various organic wastes using denaturing gradient gel electrophoresis (DGGE). Fingerprinting techniques to assess the bacterial variation was also applied such as temperature gradient gel electrophoresis

(TGGE) (Heuer and Smalla 1997). To characterize bacterial communities, Liu et al. (1997) applied the polymerase chain reaction (PCR). Amplified rDNA restriction endonucleases analysis was used by Massol-Deya et al. (1995), terminal restriction fragment length polymorphism (T-RFLP) of the 16S rRNA genes was proposed by Liu et al. (1997). Beside, Liu et al. (1997) showed that the T-RFLP fingerprinting technique has provided a rapid mean to assess community diversity in various environments. The traditional microbiological approach is still effectual (Chroni et al., 2009). El Fels et al. (2014a) in investigating the microbial succession by using two different culture approaches, Growth Standard Media (GSM) and Compost Time Extract Agar (CTEA).

Lacking knowledge about microflora, may make it difficult for engineered compost technologies to achieve desired levels of control. In order to evaluate the process and the end product biotransformation, a better knowledge of the functional microbial community dynamics is needed. The aim of this study was to investigate the microflora succession and the relation to abiotic parameters in biowaste composting, using traditional microbiological techniques.

2. SUCCESSION OF MICROORGANISMS DURING COMPOSTING

Compost microorganisms are influenced by the composition of the substrate and by the temperature in the compost pile. In addition, different microorganisms also influence each other, e.g., through competition. In the first phase of composting, microbial activity increases drastically, leading to a rise in temperature. The initial bacterial dominance is replaced by a fungal one during compost maturation. Compost management aims to achieve favorable conditions for microorganisms activity. The substrate type, the size of the compost pile and of the particles, the frequency of turning for aeration, and moistening, all these parameters affect the microbial process.

The microroganisms present at the beginning of the process are introduced with the raw matter with which the composting process is started. It has been known that composts typically contain very high numbers of microorganisms that of about 10^{10}- 10^{12} viable cells g^{-1} (Beffa et al., 1996; Tiquia et al., 1996), the major ones being bacteria (Epstein, 1997). Narihiro et al. (2004) counted up to 10^{11} cells per gram dry weight during composting of household

biowaste. Very heterogeneous groups are found in the composting substrate. Few mesophilic fungi and numerous thermophilic bacteria and fungi were found in household waste (Ryckeboer et al., 2003a). Food wastes based vegetable residues contains fungi and yeast and low bacteria concentration (Choi and Park, 1998). El Fels et al. (2015) showed that the actinobacteria and fungal microflora are the most dominant in sludge mixed with date palm waste with a dominance of mesophilic and thermotolerant microflora.

Microbial properties of the compost play a significant role in the decomposition and the humification of organic waste materials. At the beginning of the composting process, a significant change of microbial comunity occurs. The microbial biomass increases but not all microorganisms multiply at the same speed. Klamer and Baath (1998) showed a six-fold increase during the first day of composting. Microbial biomass was found to be high and its activity was essential for the release of nutrients into the medium so as to be taken by the plants (James, 1991). The indigenous microflora degrades the original substrate by producing different enzymes needed for the degradation of organic substrate, this ensures to produce metabolites and create new physical and chemical conditions during composting. These changed parameters, especially temperature, affect the succession of microbial communities (Tuomela et al., 2000).

The rise in temperature due to microbial activity at the so-called thermophilic stage affects the fungal activity which is completely suppressed (Thambirajah et al., 1995; Guo et al., 2007). Gram-positive bacteria increase with increasing temperature and decrease when the compost is cooling down (Klamer and Baath, 1998). Cunha-Queda et al. (2007) showed that during thermophilic stage the highest enzymatic activity occurs. A decrease of temperature less than 50°C is translated into a decrease in the enzymatic activity of the microbial community This characteristics determines the second important composting stage called maturation phase, during which the number of gram negative bacteria and fungi increases again (Klamer and Baath 1998). Ryckeboer et al. (2003b) showed that the bacterial and fungi diversity increases during maturation phase.

During composting, the microflora changes according to dynamic, complex microbial interactions and environmental factors. Antagonistic interactions between the different microorganisms explain also the change in microbial community.

3. MATERIAL AND METHODS

3.1. Composting Trials

Co-composting trials were conducted by mixing the activated sewage sludge/palm waste on a composting platform over a period of 180 days according to El Fels et al. (2014a, 2014b; 2015).

Mixture A: Sludge + Date palm tree waste 1:1 (v/v)

Samples of activated sewage sludge/palm waste mixture were collected at various times of composting: 0, 15, 22, 30, 60, 90 and 180 days.

Food waste was collected from the households participating in a larger study and garden clippings were composted by Chroni et al. (2009) for 125 days in a pilot plant in the Technological Education Institute (TEI) of Herakleio, Crete.

Mixture B: Food waste + Garden Clippings 1:1 (v/v).

Composite samples of approximately 1 kg were obtained on days 0, 22, 33, 40, 57, 71, 92 and 125 from three different points in the pile core.

Each mixture was carefully homogenized, ambient and pile temperatures were monitored daily. The mean value of the pile temperature at the three mixtures was noted. The mixtures were turned by hand to aerate the mixture. Water was added during turning. Homogeneous samples were taken at T_0 (first day of composting) and after each airing (aerating the mixture). Homogeneous samples (1 kg) were obtained by careful mixing of several sub samples taken at different points (height and length) of the windrow and quartering. The chemical and physical characteristics of the raw mixture and its components are presented in table 1.

3.2. Biological Analysis

3.2.1. Enumeration of Cultivable Indigenous Microflora during Sludge/Palm Composting

According to El Fels et al. (2015), the elemental samples at each composting time (0, 15, 22, 30, 60, 90 and 180 days) were first mixed,

suspended in sterile distilled water (10 g in 100 mL), homogenized by vortexing and finally treated during 10 to 15 min by sonication according to Ouhdouch et al. (2001); El Fels et al. (2014a). All treated samples were serially diluted up to 10^{-6} and functional microflora enumerated by plating and spreading 0.1 mL of 10^{-4}, 10^{-5} and 10^{-6} dilutions over the surface of the composting time extracts agar (CTEA) prepared according to El Fels et al. (2014a) as described below: one litre of distilled water and 35 g of composting time sample were mixed overnight. Agar (15 g) was added to the filtrate collected after it had been filtered and sterilized at 120°C for 15 min. The pH was adjusted to that of the composting time sample before sterilization. For each sample at the various composting times, the plates were incubated at 28°C for mesophilic and 45°C for thermophilic microflora enumeration. For fungus evaluation, the media were supplemented with 5 g/mL of chloramphenicol to inhibit the development of bacteria. For other microorganisms, the media were supplemented with 40 µg /mL of actidione to inhibit the development of fungi. Microbial analyses were performed in triplicate.

For mixture B and according to Chroni et al. (2009), microbial groups were determined by the dilution plate count technique. Mesophilic and thermophilic microorganisms were incubated at 30°C and 55°C. Total aerobic bacteria were cultivated on nutrient agar (LabM) after 2 days of incubation. Filamentous fungi were enumerated on Sabouraud dextrose agar (LabM) supplemented with Streptomycine (0.03 g/l).

Table 1. Evolution of physico-chemical parameters of substrate during composting of mixtures A and B according to El Fels et al. (2014a) and Chroni et al. (2009)

	Sewage sludge	Date palm	Food waste	Garden clippings	Mixture A		Mixture B	
					Composting time			
					T0	Tf	T0	Tf
pH	6.45	6.31	4.1	6.5	6.04	7.03	4.2	1.74
Moisture %	46.46	25.1	84.5	37.8	60.97	66	79.9	45.5
C/N	20.8	37.13	25.2	138.4	27.4	10.08	41.4	20.9

T0: Initial stage Tf: Final stage.

4. STATISTICAL ANALYSIS

The results are presented in the form of averages ± SEM (standard error of the mean). Comparison of the averages was made by ANOVA. The differences are considered significant at $p < 5\%$.

5. RESULTS AND DISCUSSION

5.1. Principal Changes of Waste Samples

Table 1 summarizes the principal chemical characteristics (average values) of the solid waste material used at the beginning of the composting process and the final composts thus obtained.

The composting process went through changes in temperature. For mixtures A (sludge and palm waste) the temperature indicated a typical composting pattern characterized by two major phases. Thermophilic phase (stabilization phase) characterized by a rise in temperature which peaked its maximum value at 65°C at 15th day of the process (El Fels et al., 2014b), thereafter at cooling stage the value decreased to reach ambient temperature. For mixture B (Food waste and Garden Clippings) the temperature remained above 50°C for about 2 months, while it reached its maximum value of 67°C at the 25th day of the process (Chroni et al., 2009). This is the result of intense microbial activity resulting from the degradation of the organic matter present in the substrate. Composting is essentially a microbiological phenomenon that depends highly on temperature fluctuation within the windrows (Hassan et al., 2002). Stentiford (1996) showed that the operating temperature ranges are as follows: >55°C to maximize sanitation, 45-55°C to maximize the biodegradation rate, and 35-40°C to maximize microbial diversity. After the thermophilic phase, the temperature of the windrow for the two mixtures A and B dropped regularly to reach ambient temperature. This second phase is due to the exhaustion of easily metabolized organic compounds from the medium with only compounds resistant to degradation remaining.

Literature data showed that the average temperature curve during composting showed two principal phases. Mesophilic microorganisms activity in waste piles tended to increase during process, which increase the temperature to reach up to 50°C as a consequence of biodegradation of organic compounds (Alberti, 1984; Mustin, 1987). This important step occurrs

generally at the first 30th days; during which the temperature exceeds the tolerance limit of mesophilic microorganisms and promotes development of thermophilic microorganisms (Hassan et al., 2002; El Fels et al., 2015). On the other hand, the increasing temperature leads to the destruction of many pathogenic microorganisms, such as coliforms, Gram-negative and Gram-positive bacteria (El Fels et al., 2016). Then the temperature decreases with the exhaustion of nutriment at second stage.

The C/N ratio drops from 27.4 to 10.08 for mixture A (sludge mixed with date palm). The C/N ratio of the raw mixture (food waste and garden clippings) was 41.7 this ratio fell to 20.9 at the 125th day (Table 1). The notable difference between C/N ration of both mixture A and B is linked to the input substrate (sewage sludge, palm waste, food waste and garden clippings), which is rich in carbon and nitrogen with significant quantities. Ryckeboer et al. (2003b) showed that carbohydrates, proteins, lipids and lignin are the main components of organic matter. At a C/N ratio between 25 and 40, the microbial enzymatic activity is intense that leads to a rise in temperature. The initial moisture content was 60.97% for mixture A and 79.9% for mixture B, the value increased to 66% for mixture A and decreased to 45% for mixture B at the end of the process. This is due to the texture of the substrates and their capacity to absorb the water. The microbial activity is influenced by the moisture content. Liang et al. (2003) showed that a minimal moisture content of about 50% is necessary for optimal aerobic activity. Nevertheless, a high moisture content affects negatively biological activity (Das and Keener, 1997) and a high water content favours bacteria over fungi (Finstein and Morris, 1975). Evaporation during composting explains also the variation rate of water content.

5.2. Microbial Succession during Composting

Microbial communities change according to temperature variations. It is important to mention that the presence of a large variety of mesophilic, thermotolerant and thermophilic aerobic microorganisms, including bacteria, actinomycetes, yeasts and various other fungi, has been extensively reported in composts and other self-heating organic materials (Amner et al., 1988; Faure and Deschamps, 1991; Finstein and Morris, 1975; Strom, 1985; Beffa et al., 1996).

During composting the indigenous microorganisms are active, they degrade the original substrate leading to a new physico-chemical environment.

This causes significant change in microbial communities (Hassen et al., 2001; El Fels et al., 2015).

The succession of microbial communities present in the compost is the result of dynamic, complex interactions between the microflora and its environment. The activity of fungal microflora is almost completely suppressed at thigh temperature (Thambirajah et al., 1995). When the compost heats the Gram-positive bacteria increase, in return however the Gram-negative bacteria and fungi increase with rising temperature, but decrease at higher temperature up to 50°C (Klamer and Baath, 1998). This explains that succession of microflora is linked to the physico-chemical changes especially temperature. Ryckeboer et al. (2003a); El Fels et al. (2014b) on the basis of phospholipid-fatty acid and fatty acid methyl-esters study showed that at end of composting most communities of microorganisms are declined. Klamer and Baath (1998) showed that fungal activity is mainly important during maturation stage.

Microbial dynamics between mixtures A and B are shown in table 2. The results show the same development appearance (profile) with a peak during the thermophilic stage of composting with higher abundance of mesophilic microflora compared to the thermophilic one. El Fels et al. (2014a) explained these high values during the thermophilic phase as a result of high levels of thermotolerant microflora in the sludge substrates. At the beginning of the composting process for mixtures A and B, the microbial activity was mainly due to the mesophilic microbial community, with total mesophilic microorganisms counts being about two and twenty orders of magnitude higher than the corresponding thermophilic group (Table 2). As shown by El Fels et al. (2014a) the mesophilic microflora (in mixture A) increased from 43 × 10^{10} CFU/g to 91 × 10^{10} CFU/g on 60 day of composting, decreasing thereafter to 56 × 10^{10} CFU/g on day 180. The thermophilic microflora varied in the range from 1.9 × 10^{10} CFU/g to 5.6 × 10^{10} CFU/g, decreasing thereafter to 1.91 × 10^{10} CFU/g on day 180 (Table 2). However in mixture B, Chroni et al. (2009) showed the total mesophilic bacteria increased from 3.2 × 10^{6} CFU/g to 1.7 × 10^{9} CFU/g on day 57. The maximum count was observed at the beginning of the cooling phase, decreasing thereafter to 3.8 × 10^{4} CFU/g on day 125. The thermophilic bacteria grew fast from 1.3 × 10^{4} CFU/g dw (dry weight) in the raw material to 1.0 × 10^{9} CFU/g dw on day 59, after the period where the highest temperatures for about 5 weeks were recorded. Their counts followed the temperature and declined thereafter, dropping to 5.1 × 10^{4} CFU/g dw on day 125 (Table 2). Particular difference was drawn between mixture A and B. The total microbial community (mesophilic and thermophilic

microflora) of activated sludge and date palm waste composting was higher than the based on food waste and garden clippings. The nature and origin of the initial substrate explain in part the differences occurring between the microbial biomass during composting of mixture A and B. Schnürer et al. (1985) showed that microbial biomass has been recognized both as a transforming agent and as a source and sink for various nutrients. The hyphal lengths and bacteria numbers increased parallel with organic matter content. The same authors showed that oxygen consumption and bacteria was twice as numerous in soil with carbon amendments as compared with control.

Fungal microflora followed the temperature variation during composting of activated sludge; they are increasing especially during the thermophilic stage of composting. They reached 50% of the total of the mesophilic and thermophilic microflora of mixtures A. The correlation between temperature and microbial succession shows that fungi and temperature are highly correlated for both mesophilic and thermophilic microflora (Figure 1). This can explain that the fungal microflora of composted substrates is especially of the thermotolerant type.

Mesophilic fungi in mixture B declined sharply as thermophilic temperatures were reached (from 2.5×10^6 to 3.2×10^2 CFU/g dw on day 22) and remained at this level thereafter. In contrast, the numbers of thermophilic fungi exhibited a small increase (from 2.8×10^2 to 9.8×10^3 CFU/g dw) as temperature rose in the first month of composting, stabilising thereafter.

The variation of the fungal population between two mixtures A and B indicates the high variation of ecological parameters during composting of both mixtures. Temperature, C/N, moisture are shown as the principal parameters which could affect the microbial succession during composting. Particular correlations (about $R^2 = 0.9$) (Figure 1) was drawn between the temperature and fungal microflora evolution during composting of sludge (mixture A). Nevertheless, there is a very low ($R^2 = 0.08$) (Figure 1) of fungal microflora and temperature correlation during composting of mixture B.

Table 2. Microbial dynamic during composting of mixture A and B according to El Fels et al. (2014a) and Chroni et al. (2009)

Mixtures	Mesophilic microflora			Thermophilic microflora		
	T0	TI	TF	T0	TI	TF
A (UFC/g)	43×10^{10}	91×10^{10}	56×10^{10}	1.9×10^{10}	56×10^{10}	1.9×10^{10}
B (UFC/g)	3.2×10^6	1.7×10^9	3.8×10^4	1.3×10^4	1.0×10^9	5.1×10^4

T0: Initial stage. TI: Intermediate stage. TF: Final stage.

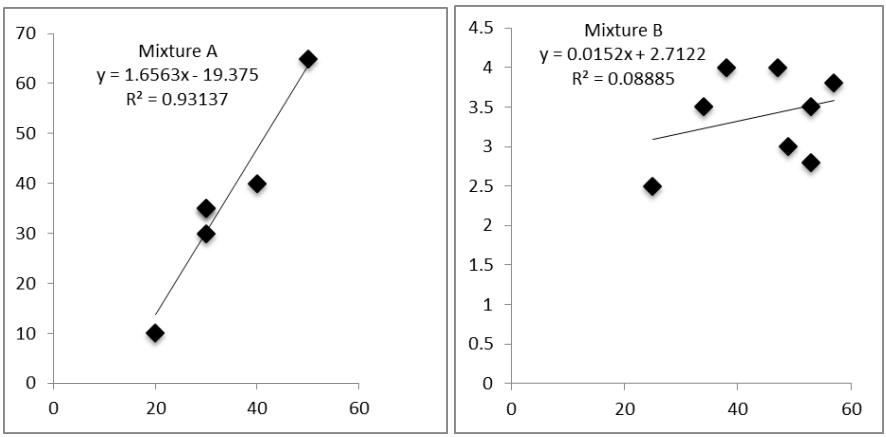

Figure 1. Correlation between fungal succession and temperature evolution during composting of sewage sludge-date palm (A) and food waste-garden clippings (B).

Finstein and Morris (1975); Ishii et al. (2000) showed that fungi proliferate especially at low temperatures (below 45°C) and maturation stage is characterized by the development of a new mesophilic fungal community. Fungi have been developed from all four phases of the compost cycle, but appear most prevalent during the initial and middle mesophilic phases (De Bertoldi et al., 1983; Ryckeboer et al., 2003a, b). Waksman et al. (1939), Ghazifard et al. (2001); Vijay et al. (2002); Anastasi et al. (2005) isolated thermotolerant fungi genera at higher temperatures. Maheshwari et al. (2000) explained that thermophilic fungi are unique group of molds that include 30 odd species with an ability to thrive at temperatures between 45°C and 60°C. The distribution, colonization and succession of thermophilic fungal population in compost is closely related with their ability to produce a variety of cell wall degrading enzymes (Sharma, 1989) including cellulases, hemicellulases and other important hydrolases, i.e., amylases, lipases, acid proteases, etc, which explain widely the proliferation of fungal microflora in mixture A based on sludge-palm composting. The above reasons explain that beside temperature the substrates and their structure is also a main key to microflora succession during composting.

CONCLUSION

The present study investigates the microbiological succession during composting of two different mixtures, mixture A based on sewage sludge-date

palm and mixture B as mixture of food waste and garden clippings. The windrow composting for biowaste progressed well, with stabilizing of the main abiotic parameters such as temperature rise and C/N ratio which reached about 10 for mixture A and 20 for mixture B. The mesophilic and thermophilic microflora varied during composting process with a peak during the thermophilic stage for both mixtures A and B. This explains that activity is due to the mesophilic, thermotolerant and thermophilic microflora. In comparison, a high microbial abundance in mixture A was noted. The fungal microflora increased highly during the thermophilic stage of composting for mixture A corresponding to 50% for the mesophilic and thermophilic microflora of mixtures. At the end of both A and B mixture composting and when easy assimilated organic compounds are consumed there is a microbial biomass declined. Several physico-chemical parameters influence the microbial change during composting, and one of the most important factors in this biological process seems to be temperature and the nature of the initial substrate.

REFERENCES

Alberti, G. (1984). Aspects bactériologiques du compostage des boues résiduaires de stations d'épuration d'eau. Thèse de 3ème Cycle. Université de Nancy I, France, 200p.

Amner, W., McCarthy, A. J., and Edwards, C. (1988). Quantitative assessment of factors affecting the recovery of indigenous and release thermophilic bacteria from compost. *Applied Environmental Microbiology*. 54, 3107-3112.

Anastasi, A., Varese, G. C., Marchisio, V. F. (2005). Isolation and identification of fungalcommunities in compost and vermicompost. *Mycologia.* 97 (1), 33–44.

Beffa, T., Blanc, M., Marilley, L., Lott Fisher, J., and Lyon, P. F. (1996). Taxonomic and metabolic microbial diversity during composting. In The Sciences of Composting, De Bertoldi, M., Sequi, P., Lemmes, B., Papi, T., eds., Blackie Academic and Professional, Glasgow, UK, pp. 149-161.

Choi, M. H., Park, Y. H. (1998). The influence of yeast on thermophilic composting of food waste. *Letters in Applied Microbiology*. 26(3), 175-178.

Chroni, C., Kyriacou, A., Georgaki, I., Manios, T., Kotsou, M., Lasaridi, K. (2009): Microbial characterization during composting of biowaste. *Waste Management.* 29, 1520–1525.

Cunha-Queda, A. C., Ribeiro, H. M., Ramos, A., Cabral, F. (2007). Study of biochemical and microbiological parameters during composting of pine and eucalyptus bark. *Bioresource Technology*. 98(17), 3213-3220.

Curiale, M. S. and Klatt, M. J. (1990). Colorimetric deoxyribonucleic acid hybridization assay for rapid screening of Salmonella in foods: Collaborative Study. *Journal-Assoc Of official Analytical Chemists*. 73, 248-256.

Das, K., Keener, H. M. (1997). Moisture effect on compaction and permeability in composts. *Journal of environmental engineering*. 123(3), 275-281.

De Bertoldi, M., Vallini, G., Pera, A. (1983). The biology of composting: a review. *Waste Management Resources.* 1, 157–176.

El Fels, L., Ouhdouch, Y., Hafidi, M. (2015). Use of the co-composting time extract agar to evaluate the microbial community changes during the co-composting of activated sludge and date palm waste. *International Journal of Recycling of Organic Waste in Agriculture*. 4(2), 95-103.

El Fels, L., Hafidi, M., Ouhdouch, Y. (2016). Date palm and the activated sludge co-composting actinobacteria sanitization potential. *Environmental Technology*. 37(1), 129-135.

El Fels, L., El Ouaqoudi, F. Z., Barje, F., Hafidi, M., Ouhdouch, Y. (2014a). Two culture approaches used to determine the co-composting stages by assess of the total microflora changes during sewage sludge and date palm waste co-composting. *Journal of Environmental Health Science and Engineering.* 12(1), 1.

El Fels, L., Zamama M., El Asli, A., Hafidi, M. (2014b) Assessment of biotransformation of organic matter during co-composting of sewage sludge- lignocelullosic waste by chemical FTIR analyses and phytotoxicity tests. *International Biodeterioration and Biodegradation*. 87, 128–137.

Epstein, E. (1997) The science of composting Lancaster. *Technomic Publishing Company*, Lancaster.

Faure, D. and Deschamps, A. M. (1991). The effect of bacterial inoculation on the initiation of composting grape pulps. *Bioresource Technology*. 37, 235-238.

Finstein, M. S., and Morris, M. L. (1975). Microbiology of municipal solid waste composting. *Advances in Applied Microbiology*. 19, 113-153.

Gharib, F. A., Moussa, L. A., Massoud, O. N. (2008). Effect of compost and bio-fertilizers on growth, yield and essential oil of sweet marjoram

(Majorana hortensis) plant. *International Journal of Agriculture and Biology*. 10(4), 381-382.

Ghazifard, A., Kasra-Kermanshahi, R., Far, Z. E. (2001). Identification of thermophilic and mesophilic bacteria and fungi in Esfahan (Iran) municipal solid waste 455 compost. *Waste Management and Research.* 19 (3), 257–261.

Guo, Y., Zhu, N., Zhu, S., Deng, C. (2007). Molecular phylogenetic diversity of bacteria and its spatial distribution in composts. *Journal of Applied Microbiology.* 103(4), 1344-1354.

Hassen, A., Belguith, K., Jedidi, N., Cherif, A., Cherif, M., Boudabous, A. (2001) Microbial characterization during composting of mu- nicipal solid waste. *Bioresource Technology.* 80, 217–225.

Heuer, H., Smalla, K., van Elsas, J. D., Trevors, J. T., Wellington, E. M. (1997). Application of denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis for studying soil microbial communities. *Modern Soil Microbiology*. 353-373.

Ishii, K., Fukui, M., Takii, S. (2000). Microbial succession during a composting process as evaluated by denaturing gradient gel electrophoresis analysis. *Journal Of Applied Microbiology*. 89,768–777.

James, S. W. (1991). Soil nitrogen, phosphorus and organic matter processing by earthworms in tall grass prairie. *Ecology*. 72, 2101-2109.

Klammer, M., Baath, E. (1998). Microbial community dynamics during composting of straw material studied using phospholipid fatty acid analysis. *FEMS Microbiology Ecology.* 27, 9–20.

Klammer, S., Knapp, B., Insam, H., Dell'Abate, M. T., Ros, M. (2008). Bacterial community patterns and thermal analyses of composts of various origins. *Waste Management Resources.* 26, 173–187.

Krieger, J. (1992) Technique Identifies Bacteria that Degrade Wastes. Science/ Technology p36.

Liang, C., Das, K. C., McClendon, R. W. (2003). The influence of temperature and moisture contents regimes on the aerobic microbial activity of a biosolids composting blend. *Bioresource Technology*. 86(2), 131-137.

Liu, W. T., Marsh, T. L., Cheng, H., Forney, L. J. (1997). Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Applied and Environmental Microbiology.* 63, 4516–4522.

Maheshwari, R., Bharadwaj, G. and Bhat, M. K. (2000) Thermophilic fungi: their physiology and enzymes. *Microbiology and Molecular Biology Reviews.* 64, 461–488.

Mustin, M. (1987). Le compost, gestion de la matière organique. François Dubusc, Paris, p. 954.

Narihiro, T., Abe, T., Yamanaka, Y., Hiraishi, A. (2004). Microbial population dynamics during fed-batch operation of commercially available garbage composters. *Applied Microbiology and Biotechnology*. 65(4), 488-495.

Riddech, N., Klammer, S., Insam, H. (2002). Characterisation of Microbial Communities during Composting of Organic Wastes. In Microbiology of Composting. Edited by Insam H, Riddech R, Klammer S. Heidelberg: Springer Verlag: 43–45.

Ryckeboer, J., Mergaert, J., Coosemans, J., Deprins, K., Swings, J. (2003a). Microbiological aspects of biowaste during composting in a monitored compost bin. *Journal of Applied Microbiology*. 94(1), 127-137.

Ryckeboer, J., Mergaert, J., Vaes, K., Klammer, S., De Clercq, D., Coosemans, J., Insam, H., Swings, J. (2003b). A survey of bacteria and fungi occurring during composting and self-heating processes. *Annals of Microbiology.* 53(4), 349-410.

Schnürer, J., Clarholm, M., Rosswall, T. (1985). Microbial biomass and activity in an agricultural soil with different organic matter contents. *Soil Biology and Biochemistry*. 17(5), 611-618.

Sharma, H. S. K. (1989) Economic importance of thermophilous fungus. *Applied Microbiology and Biotechnology.* 31, 1–10.

Stentiford, E. I. (1996). Composting control: Principles and Practice. In The Sciences of Composting, De Bertoldi, M., Sequi, P., Lemmes, B., Papi, T., eds., Blackie Academic and Professional, Glasgow, UK, pp. 49-59.

Strom, P. F. (1985). Effect of temperature on bacterial diversity in thermophilic solid-waste composting. *Applied and Environmental Technology*. 50, 906-913.

Thambirajah, J. J., Zulkali, M. D., Hashim, M. A. (1995). Microbiological and biochemical changes during the composting of oil palm empty-fruit-bunches. Effect of nitrogen supplementation on the substrate. *Bioresource Technology*. 52(2), 133-144.

Tiquia, S. M., Tam, N. F. Y., Hodgkiss, I. J. (1996). Effect of composting on phyto-toxicity of spent pig manure saw dust litter. *Environmental Pollution*. 93, 249-256.

Tuomela, M., Vikman, M., Hatakka, A., Itavaara, M. (2000). Biodegradation of lignin in a compost environment: a review. *Bioresource Technology.* 72:169–183.

Vijay, B., Sharma, S. R., Lakhanpal, T. N. (2002). Role of thermophilic fungi in compost production for Agaricus bisporus. *Journal of Mycology and Plant Pathology*. 32, 513 204–210.

Waksman, S. A., Umbreit, W. W., Cordon, T. C. (1939). *Thermophilic actinomycetes and fungi in soils and in composts. Soil* Science. 47 (1), 37–61.

BIOGRAPHICAL SKETCH

Loubna El Fels

Affiliation: Professor

Education: Professor at Higher Institute of Nursing Professions and Health Technics, Marrakech-Safi, Morocco

Business Address: Avenue Moulay Abdellah Al Amir, Safi-Morocco

Research and Professional Experience: Composting process, Microbial dynamic, waste management, soil, Agriculture, Environmental science.

Publications:

1. Lahsaini, S., Idrissi, L., Souabi, S., El Fels, L., Aguelmous, A. and Hafidi, M. (2016). Comparative study of organic matter degradation of composted sludge and sludge landfilled/etude comparative de la degradation de la matiere organique des boues compostees et boues mises en decharge. Scientific Study and Research. *Chemistry and Chemical Engineering, Biotechnology, Food Industry*, 17(1), 1.
2. El Fels, L., Lemee, L., Ambles, A. and Hafidi, M. (2016). Identification and biotransformation of aliphatic hydrocarbons during co-composting of sewage sludge-Date Palm waste using Pyrolysis-GC/MS technique. *Environmental Science and Pollution Research*, 1-8.
3. El Fels, L., Hafidi, M. and Ouhdouch, Y. (2016). Artemia salina as a new index for assessment of acute cytotoxicity during co-composting of sewage sludge and lignocellulose waste. *Waste Management*, *50*, 194-200.
4. El Fels, L., Hafidi, M. and Ouhdouch, Y. (2016). Date palm and the activated sludge co-composting actinobacteria sanitization potential. *Environmental technology*, *37*(1), 129-135.
5. El Fels, L., El Ouaqoudi, F. Z., Lemme, L., Geffroy, C., Ambles, A. and Hafidi, M. (2016). Occurrence of plant and fecal steroid and their

evolution during co-composting of sewage sludge and lignocellulosic waste. *Biochemical Engineering Journal*, *105*, 497-504.

6. Benlboukht, F., El Fels, L., Bouamri, R., Amir, S. and Hafidi, M. (2016). Assessment of Fulvic Acid-Like Fractions during Tannery Waste Composting. Compost Science and Utilization, 24(3), 208-218.
7. El Fels, L., Ouhdouch, Y. and Hafidi, M. (2015). Use of the co-composting time extract agar to evaluate the microbial community changes during the co-composting of activated sludge and date palm waste. *International Journal of Recycling of Organic Waste in Agriculture*, *4*(2), 95-103.
8. El Fels, L., Hafidi, M., Silvestre, J., Kallerhoff, J., Merlina, G. and Pinelli, E. (2015). Efficiency of co-composting process to remove genotoxicity from sewage sludge contaminated with hexavalent chromium. *Ecological Engineering*, *82*, 355-360.
9. El Ouaqoudi, F. Z., El Fels, L., Lemée, L., Amblès, A. and Hafidi, M. (2015). Evaluation of lignocelullose compost stability and maturity using spectroscopic (FTIR) and thermal (TGA/TDA) analysis. *Ecological Engineering*, *75*, 217-222.
10. Elouaqoudi, F. Z., El Fels, L., Amir, S., Merlina, G., Meddich, A., Lemee, L., Ambles, A. and Hafidi, M. (2015). Lipid signature of the microbial community structure during composting of date palm waste alone or mixed with couch grass clippings. *International Biodeterioration and Biodegradation*, *97*, 75-84.
11. El Faiz, A., El Fels, L., Meddich, A., Ouhammou, A., Taourirte, M. and Hafidi, M. (2015). Assessment of Biodegradation of Oleandrin during Co-composting of Nerium oleander L. Wastes. *Compost Science and Utilization*, *23*(1), 37-47.
12. El Fels, L., El Ouaqoudi, F. Z., Lemée, L., Winterton, P., Merlina, G., Ouhdouch, Y., E. Pinelli, A. Ambles and Hafidi, M. (2015). Identification and assay of microbial fatty acids during co-composting of active sewage sludge with palm waste by TMAH-thermochemolysis coupled with GC-MS. *Chemistry and Ecology*, *31*(1), 64-76.
13. El Ouaqoudi, F. Z., El Fels, L., Winterton, P., Lemée, L., Amblès, A. and Hafidi, M. (2014). Study of humic acids during composting of ligno-cellulose waste by infra-red spectroscopic and thermogravimetric/ thermal differential analysis. *Compost Science and Utilization*, 22(3), 188-198.

14. El Fels, L., Zamama, M., El Asli, A. and Hafidi, M. (2014). Assessment of biotransformation of organic matter during co-composting of sewage sludge-lignocelullosic waste by chemical, FTIR analyses, and phytotoxicity tests. *International Biodeterioration and Biodegradation*, *87*, 128-137.
15. El Fels, L., Lemee, L., Ambles, A. and Hafidi, M. (2014). Identification and biotransformation of lignin compounds during co-composting of sewage sludge-palm tree waste using pyrolysis-GC/MS. *International Biodeterioration and Biodegradation*, *92*, 26-35.
16. El Fels, L., El Ouaqoudi, F. Z., Barje, F., Hafidi, M. and Ouhdouch, Y. (2014). Two culture approaches used to determine the co-composting stages by assess of the total microflora changes during sewage sludge and date palm waste co-composting. *Journal of Environmental Health Science and Engineering*, *12*(1), 1.

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