Advances in CLINICAL CHEMISTRY

VOLUME 46

Edited by

Gregory S. Makowski

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Academic Press is an imprint of Elsevier

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32 Jamestown Road, London, NW1 7BY, UK
Radarweg 29, PO Box 211, 1000 AE Amsterdam, The Netherlands
30 Corporate Drive, Suite 400, Burlington, MA 01803, USA
525 B Street, Suite 1900, San Diego, CA 92101-4495, USA

This book is printed on acid-free paper. ♾

British Library Cataloguing Publication Data
A catalogue record for this book is available from the British Library.

Library of Congress Cataloging-in-Publication Data
A catalog record for this book is available from the Library of Congress.

ISBN: 978-0-12-374209-4
ISSN: 0065-2423

For information on all Academic Press publications
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Printed and bound in USA
08 09 10 11 12 10 9 8 7 6 5 4 3 2 1

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PREFACE

I am pleased to present volume forty-six of *Advances in Clinical Chemistry* series for the year 2008.

In this second volume for the year, a diverse array of topics is presented. The role of low-density lipoprotein particle size and number is explored in relationship to coronary heart disease risk stratification. In this chapter, methodologies available for atherogenic lipid subprofiling are also discussed. The effect of exercise on oxidative stress as well as their effect on upregulation of endogenous antioxidant defense systems is explored in another chapter. A comprehensive chapter on the serum N-glycome is presented. These modified proteins may present novel targets to identify congenital and acquired disorders. Nutritional biomarkers associated with adaption to microgravity are highlighted in another chapter. Identifying the role of these nutrients in maintenance of muscle, bone, and cardiovascular systems during prolonged space flight is crucial as we approach the next frontier in our exploration of the cosmos. An interesting review is also presented on the use of specific biomarkers in lieu of biopsy in chronic liver disease. A chapter on biomarkers of aging discusses their use in population research and their relationships to health outcomes. Finally, we conclude with two excellent chapters that address calcification processes in general and the impact of this pathophysiological process in renal disease specifically.

I extend my appreciation to each contributor of volume forty-six and thank colleagues who contributed to the peer review process. I extend thanks to my Elsevier editorial liaison, Ms Pat Gonzalez for her continued support.

I sincerely hope the second volume of 2008 will be enjoyed by our readership. As always, I warmly invite comments and suggestions for future review articles for the *Advances in Clinical Chemistry* series.

In keeping with the tradition of the series, I would like to dedicate volume forty-six to my father Edmund who recently passed.

GREGORY S. MAKOWSKI

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EFFECT OF EXERCISE ON OXIDATIVE STRESS BIOMARKERS

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1. Abstract

Acute bouts of aerobic and anaerobic exercise can induce a state of oxidative stress, as indicated by an increase in oxidized molecules in a variety of tissues and body fluids. The extent of oxidation is dependent on the exercise mode, intensity, and duration, and is specifically related to the degree of oxidant production. Findings of increased oxidative stress have been reported for both healthy and diseased subjects following single bouts of exercise. While acute exercise has the ability to induce an oxidative stress, this same exercise stimulus appears necessary to allow for an upregulation in endogenous antioxidant defenses. This chapter presents a summary of exercise-induced oxidative stress.

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0065-2423/08 $35.00
DOI: 10.1016/S0065-2423(08)00401-0

2. Introduction

The topic of exercise-induced oxidative stress has received considerable attention in recent years, with more than 300 original investigations published since Dillard *et al.* first reported in 1978 that lipid peroxidation was increased following 60 min of cycling exercise [1]. Based on the available evidence, it appears clear that exercise of sufficient intensity and duration increases the formation of reactive oxygen and nitrogen species (RONS), having the potential to create an imbalance between oxidant and antioxidant levels. This condition, commonly referred to as oxidative stress, can lead to the oxidation of lipids, proteins, deoxyribonucleic acid (DNA), and other molecules. The degree of oxidation as well as the time course for elevation in oxidative stress biomarkers varies across studies, and likely depends on the type, intensity, volume and duration of exercise, the exercise training and nutritional status of the research subjects, the time course of sample collection, the tissue being investigated, and the various assays employed. While acute exercise appears to increase oxidative stress transiently, this same exercise stimulus is necessary to allow for an upregulation in endogenous antioxidant defenses. In this way, the generation of RONS appears to be the "signal" needed to allow for such important adaptations, which very likely protect cells from future elevations in RONS. This chapter presents a summary of exercise-induced oxidative stress by first providing an overview of RONS formation and methods of assessment, as well as protective mechanisms against RONS attack. Results from both acute aerobic and anaerobic exercise studies are discussed, as well as adaptations to the antioxidant defense system as a result of chronic exercise training. More specific aspects of exercise-induced oxidative stress such as gender, age, and disease status differences are also highlighted.

3. Description of Oxidative Stress

Oxidative stress is a condition in which an imbalance exists between oxidant and antioxidant levels in such a way that oxidant production overwhelms antioxidant defenses, often leading to oxidation of lipids, proteins, DNA, and other molecules in ways that impair cellular function [2]. The generation of RONS occurs in part as a consequence of normal cellular metabolism [3]. Under ordinary physiological conditions, the body's endogenous antioxidant defense system, in conjunction with exogenous antioxidants consumed through dietary sources, acts to protect small and macromolecules from modification and destruction via oxidants.

In addition to normal cellular metabolism, oxidants can be generated through exposure to a wide variety of environmental (e.g., cigarette smoke, ozone, certain nutrients) as well as physiological (e.g., physical and mental stress) challenges. Specifically, oxidative stress may be mediated by an increased activity of radical-generating enzymes (e.g., xanthine oxidase); activation of phagocytes, phospholipases, cyclooxygenases, and lipoxygenases; release of heme proteins via destruction of iron-containing proteins; through disruption of the electron transport system leading to increased leakage of superoxide radicals; and via suppressed antioxidant protection [4]. Specific information pertaining to sources of RONS generation is presented in Section 3.3.1.

3.1. Associations with Health and Disease

During and following stressful conditions in which RONS production is increased, adequate protection may not be available, and the capacity of the antioxidant defense system may be overwhelmed by the oxidant attack. Such conditions can lead to progressive oxidation of cellular components such as lipids, proteins, and nucleic acids, and have been implicated in a wide variety of disease processes, as well as in aging [5]. In many cases, the evidence consists only of observations of increased levels of oxidative stress biomarkers (e.g., lipid, protein, DNA oxidation) in persons with a particular disease state, such as cardiovascular disease [6] and diabetes [7], which is considered to merely represent an association rather than a causal role. In other cases, subjects with certain diseases such as type II diabetes [8], hypertension [9], heart failure [10], and coronary artery disease [11] demonstrated improved markers of health following antioxidant therapy, suggesting that oxidant stressors may have been one possible cause of disease or of disease progression.

While numerous studies have found that an association exists between elevated oxidative stress and disease (for review please see Ref. [12]), specific cause and effect data are more scarce. Regardless, this area of study continues to be an important focal point of ongoing health-related research. This is because a myriad of diseases appear to have strong correlations to increased concentrations of RONS in both tissue and blood though it is understood that the complexity of most disease processes certainly does not exclude the probability that factors outside of RONS also play a key role. Indeed, most pathologies are multifactorial. Additional study is needed before firm conclusions can be made pertaining to whether oxidants are a major cause of disease or merely a consequence of the disease process. With this understanding, at least some direct evidence exists linking oxidative stress to both the initiation and progression of atherogenesis [13], diabetes [14], cancer [15], respiratory ailments [16], and neurodegenerative disorders [17]. Moreover, oxidative stress appears to expedite the aging process [18].

Despite the potential problems associated with excessive oxidants, it is well understood within the scientific community that RONS are involved in several important biological processes. In particular, RONS play important roles in cell signaling [19], redox regulation of gene transcription [20], cellular immunity [21], and apoptosis [22]. Thus, RONS are essential for normal physiological function, and "healthy" levels that do not exceed a certain unknown threshold do not appear problematic.

3.2. Association with Physical Performance (Muscle Function)

Animal studies have noted altered contractile function, reductions in muscle force output, and greater fatigue rates as a function of increased oxidative stress in isolated skeletal muscle [23]; however, little direct evidence is available linking oxidative stress to impaired physical performance in humans. It is conceivable that RONS-mediated oxidative damage to cellular components can indeed impact muscle performance in a negative way. This is particularly true with regards to proteins, as both contractile (primarily myosin due to thiol group oxidation [24]) and enzymatic proteins may be oxidized by RONS, leading to problems with excitation–contraction coupling [25] and the potential for slowing reaction rates, respectively. Clearly, mitochondrial enzymes required for energy production (e.g., succinate dehydrogenase, cytochrome oxidase) are susceptible to oxidation [26]. Oxidative damage to adenosine triphosphatase pumps can decrease calcium reuptake by the sarcoplasmic recticulum, leading to an imbalance in calcium homeostasis [27] and reduced muscle contractility. Moreover, oxidation of ryanodine receptors regulating calcium release channels in the sarcoplasmic recticulum leads to excess calcium availability and promotes muscle contractures [28].

Several studies have noted an elevation in oxidative stress biomarkers following strenuous exercise, which often correlates to reductions in muscle force, as well as increased muscle soreness—leading to the hypothesis that oxidative stress is responsible for the force loss and increased soreness in the hours to days following exercise. However, it is difficult to state with certainty that the increased oxidative stress actually caused the impairments in muscle performance and muscle soreness. More work is needed in this area of study before definite conclusions can be drawn regarding the role of RONS in impaired muscle functioning.

Related to the above discussion, a few studies have investigated the link between oxidative stress and excessive exercise (i.e., overtraining). Conditions of aerobic overtraining (4 weeks) have been linked to decreased blood antioxidant status in humans [29], while a shorter period of overtraining (3 weeks) was recently associated with increased lipid peroxidation in animals [30]. Similar findings for increased serum total peroxides have been

noted in professional football players during the course of the 5-month competitive season [31]. Despite these observations, specific measures of performance were not investigated along with the assessment of oxidative stress biomarkers in these studies. We have recently found that short-term anaerobic overtraining (7 days: two sessions per day of squat exercise) does not impair protein oxidation but does impair exercise performance (unpublished data). It is possible that different forms of exercise as well as different lengths of overtraining can lead to varying degrees of oxidative stress and impaired physical performance. Additional work using human subjects is needed in this area, with the inclusion of both oxidative stress and performance measures within the same study design.

3.3. Reactive Oxygen and Nitrogen Species

As related to the above and as discussed earlier, oxidative stress results when RONS production exceeds antioxidant defense. RONS include both nonradical and radical species, the latter often referred to as "free radicals". Electrons within atoms and molecules occupy regions of space known as orbitals, each holding a maximum of two electrons. Most biological molecules are nonradicals, containing only paired electrons. However, if a single electron is unpaired in an orbital, it is said to be free, and a radical can be defined as any species capable of independent existence containing one or more unpaired electrons [32]. The unpairing and formation of free radicals can occur by three different mechanisms: (1) Homolysis of covalent bonds: $A - B \rightarrow A^{\bullet} + B^{\bullet}$; (2) addition of a single electron (e) to a neutral atom: $A + e \rightarrow A^{\bullet\,-}$; (3) loss of a single electron (e) from a neutral atom: $A \rightarrow A^{+\bullet} + e$ [33]. Free radicals are generally regarded as highly reactive because they seek to accept electrons from other molecules. The donation of electrons can thus produce additional free radicals, leading to a chain reaction of free radical generation, which continues until a chain terminating reaction occurs. A listing of common RONS is provided in Table 1, where it should be noted that the term RONS includes both radical and nonradical species.

3.3.1. *Generation of RONS*

Multiple potential sites exist for RONS formation in relation to an acute bout of exercise. These include both primary sources in which RONS are generated in direct response to a given condition, and secondary sources in which RONS production may occur in response to damage induced through other mechanisms (e.g., eccentric muscle actions). Simple measurement of oxidative stress biomarkers does not allow for the differentiation between primary and secondary RONS production. However, measuring multiple samples over time may provide some insight into potential sources of

TABLE 1
COMMON REACTIVE OXYGEN AND NITROGEN SPECIES

Reactive oxygen species	
Singlet oxygen	$^1O_2$
Superoxide	O_2^-
Hydrogen peroxide	H_2O_2
Hydroxyl radical	$OH^\bullet$
Perhydroxyl radical	$HO_2^\bullet$
Alkoxyl radical	$RO^\bullet$
Peroxyl radical	$ROO^\bullet$
Hydroperoxyl radical	$ROOH^\bullet$
Hypochlorous acid	HOCL
Ozone	O_3
Reactive nitrogen species	
Nitric oxide	NO
Nitric dioxide	NO_2
Peroxynitrite	ONO_2^-

generation (i.e., respiratory chain leakage of superoxide during acute exercise vs phagocytic respiratory burst production of radical species following exercise in response to muscle injury).

3.3.1.1. *Primary Sources.* A major pathway for radical generation in biological systems involves mitochondrial electron transport, where oxygen (O_2) is used for ATP production. Under normal physiological conditions, most of the oxygen consumed by cells is reduced to water in the mitochondria through a series of one-electron reductions via the action of the cytochrome oxidase complex. However, a small amount (1–5%) of the oxygen passing through the respiratory chain may give rise to superoxide, leading to other nonradical (e.g., hydrogen peroxide) and radical species (e.g., hydroxyl radical). This may stem from the one-electron reduction of ubiquinone (coenzyme Q), generating ubisemiquinone, which subsequently leaks its unpaired electron to oxygen to form superoxide. However, there may be other generating sites of free radicals in the electron transport chain [34].

Mitochondria also generate nitric oxide (NO) [35], which may react with superoxide to form peroxynitrite (ONO_2^-), a very potent oxidant. It follows that increased respiration with enhanced flow of electrons in electron transport, as during an acute bout of exercise in which oxygen uptake may increase 10- to 20-fold, may increase RONS production. The mitochondria are well defended against superoxide radical-mediated damage through the action of superoxide dismutase (SOD); however, in isolated mitochondrial fractions, marked damage may be evident from long-lasting exercise [36]. Furthermore, while it is generally believed that the increase in RONS

through the mitochondria is specific to electron leakage, it has been proposed that drastic changes in temperature during exercise [36], in addition to a decreased mitochondrial PO_2 [37], could also be major mechanisms to increase RONS.

In addition to generation through mitochondrial electron transport, RONS can be produced through prostanoid metabolism. Prostaglandins are released from cells within muscle in response to an acute stressor (e.g., excessive contractile activity), the intermediates being RONS. The precursor of prostaglandins, arachidonic acid, can be converted to active metabolites by lipoxygenase, producing additional radical species. Catecholamines, which are increased during exercise, can auto-oxidize leading to RONS production or undergo metal ion-catalyzed oxidation to free radical species [38].

The enzymes xanthine oxidase and NADPH oxidase have also been noted to be radical species generators [33, 38]. Depleted levels of ATP, often mediated by strenuous exercise involving an acute state of ischemia (followed by reperfusion), lead to high intracellular levels of ADP, which promote ADP degradation and conversion of xanthine dehydrogenase to xanthine oxidase, a superoxide radical generator. The formation of xanthine oxidase usually occurs in the presence of hypoxanthine, which is generated via the degradation of nucleotides in muscle, in addition to the activation of calcium-dependent proteases, which increase when calcium homeostasis is compromised (i.e., as a result of exercise-induced muscle injury). Thus, generation of RONS via the xanthine oxidase pathway likely involves high-intensity, short-duration exercise conditions where muscle is metabolically compromised and perhaps damaged (i.e., ATP degradation is greater than generation and calcium homeostasis is lost).

NADPH oxidase, present in neutrophils and other cell types, can also act as a site for RONS production. However, at present it is uncertain if this enzyme is present in skeletal muscle or whether it is influenced by contractile activity. Therefore, the possibility that it acts as a contributor to RONS production is largely speculative.

3.3.1.2. *Secondary Sources.* Other sources of RONS generation can arise from exercise which leads to tissue injury (e.g., high force eccentric muscle actions). First, invasion of phagocytic cells (e.g., neutrophils, monocytes, eosinophils, macrophages) into damaged tissue, perhaps as a result of an acute insult, can generate a substantial amount of reactive oxygen species including superoxide and hydrogen peroxide. In this way, the radicals serve a useful purpose in degrading severely damaged tissue so that regeneration can occur.

The disruption of iron-containing proteins such as erythrocytes and myoglobin can lead to an increase in free iron, which is known to catalyze radical reactions. Exercise that creates a significant degree of trauma (e.g., high force eccentric muscle actions, high impact aerobic exercise) may lead to

destruction of these proteins, allowing for increased free iron availability to aid in the production of RONS. Moreover, anaerobic exercise promotes acidosis and excess lactate accumulation leading to iron release from transferrin [39] and the potential for the conversion of superoxide to hydroxyl radical. Relevant to iron-containing proteins, the increased oxidation of hemoglobin and myoglobin that occurs with intense exercise can cause RONS formation. Hemoglobin auto-oxidation produces methemoglobin and superoxide [40], while myoglobin auto-oxidation generates hydrogen peroxide [41], which can later react to form more harmful oxidants. The generation of RONS may also be associated with an imbalance in calcium homeostasis. Here, excessive intracellular calcium accumulation may lead to RONS production through activation of phospholipase and proteolytic enzymes.

In the above ways, generation of RONS can be increased as a result of an exercise stimulus. The increase may be a result of increased oxygen consumption to fuel muscular work, or via any number of other primary or secondary sources of radical species generation. Due to the variety of RONS-generating pathways, increased oxidative stress has been observed following both aerobic and anaerobic exercise bouts. Because it is unknown whether the observation of increased RONS is a direct result of the exercise stimulus or whether the increase is secondary to the initial insult, caution should be used when interpreting data. Conclusively stating that the radicals were generated by one mechanism vs another may be difficult to substantiate, especially in exercising humans. In fact, it is likely that RONS production involves several pathways, all of which collectively lead to the presence of oxidized molecules in biological sample analysis. Figure 1 presents a schematic of the potential mechanisms by which an acute exercise bout could induce RONS production.

3.3.2. *Types of Radicals Generated*

Several distinct RONS exist in biological systems, with the most widely discussed and investigated presented in this section. Superoxide radical ($O_2^{\bullet -}$) is the one-electron reduction product of molecular oxygen and is considered to be highly reactive when dissolved in organic solvents. It is formed mainly through singlet oxygen ($^1O_2$) within the mitochondrial electron transport chain, where singlet oxygen can bind to molecular oxygen to form a superoxide radical. This singlet oxygen is produced mainly from mitochondrial leakage during oxidative phosphorylation [42], and itself appears more reactive toward protein than it is to lipids or DNA [32]. Superoxide radicals can also be generated through elimination reactions from peroxyl radicals, through electron transfer reactions from other free radicals [4], and through the respiratory burst of phagocytic cells such as neutrophils, monocytes,

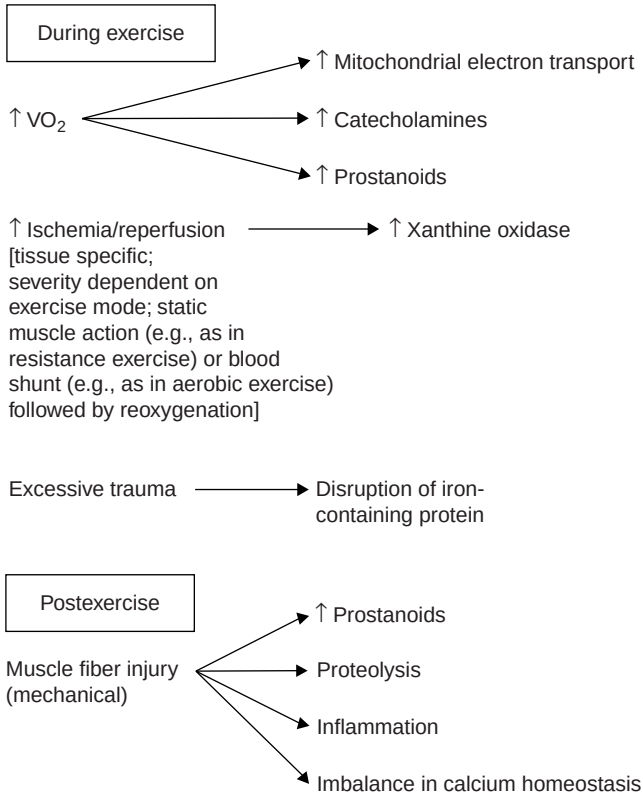

FIG. 1. Potential mechanisms of increased RONS production related to an acute bout of exercise. Adapted with permission from Bloomer and Goldfarb [2].

eosinophils, and macrophages [43]. Specific to the immune response through phagocytic cells, production of superoxide radical is viewed as beneficial, since invading bacteria can be engulfed and destroyed by antibacterial mechanisms, including lysosomal enzymes and myeloperoxidase.

In aqueous solutions, the superoxide radical is poorly reactive; however, systems producing it can do a great deal of damage *in vitro* (e.g., they fragment DNA and polysaccharides, kill bacteria and animal cells in culture) and *in vivo* (e.g., produce inflammation and cell damage upon tissue-specific infusion in animals). The potential harm with this radical within free living biological systems lies in its ability to be converted into other radical-generating species such as hydrogen peroxide (H_2O_2), while first combining with a proton to yield hydroperoxyl radical ($ROOH^{\bullet}$).

Hydrogen peroxide can be produced, as depicted above, from superoxide radicals (by SOD), in addition to nonenzymatic production and direct production via a number of oxidase enzymes. However, hydrogen peroxide is not itself considered an oxygen free radical, but has the ability to induce oxidative stress by activating oxidant-generating enzymes [44]. As with superoxide radical, much of the cytotoxic effects of hydrogen peroxide are due to its ultimate conversion into the hydroxyl radical ($OH^{\bullet}$), the most reactive oxygen-containing species known. This conversion can occur in the presence of reduced transition metals, such as copper and iron, via the Fenton reaction as follows:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$

Additionally, hydroxyl radical can be formed by the interaction of superoxide with hydrogen peroxide through the Haber-Weiss reaction as follows:

$$O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + OH^{\bullet} + OH^{-}$$

With a rise in hydroxyl radicals, the potential exists for damage to various cellular structures. This is because the hydroxyl radical reacts with virtually every structure from which it can abstract an electron, including small molecules such as glucose or macromolecules such as proteins, lipids, and DNA, often giving rise to a chain reaction [45]. This occurs at a diffusion-controlled rate (i.e., hydroxyl radicals react as soon as they come into contact with another molecule in solution [46]). Generally, cellular modification/damage is specific to the immediate vicinity of hydroxyl radical production due to such high reactivity [47]. Therefore, generation within cell membrane may lead to damage of polyunsaturated fatty acids comprising the lipid bilayer, while production in proximity to proteins or nucleic acids may promote enzymatic or functional amino acid damage, in addition to modifications in nucleotide bases. Other radical species may be formed due to modifications in proteins or polyunsaturated fatty acids by hydroxyl radicals and lead to cellular damage.

While hydroxyl radicals may be the most reactive in biological systems, peroxyl radicals are the most abundant, as they are formed in any oxygen-containing environment. Specifically, organic peroxyl radicals ($ROO^{\bullet}$) result from oxygen addition to virtually any carbon-centered free radical ($R^{\bullet}$) and can act on electron transfer and addition reactions as well as resemble similar reactivity features as hydroxyl radicals. The chain reaction sequence in lipid peroxidation, which has been noted to damage cellular membranes, is an important consequence of the hydroxyl radicals.

Aside from oxygen-centered free radical species, nitrogen-based molecules can react with transition metals or with other radicals (e.g., superoxide) to form harmful agents. Nitric oxide can react with transition metals to form

metal-nitrosyl adducts, with superoxide radicals to form peroxynitrite, a highly toxic compound, as well as with other oxygen-centered radicals to form reactive nitrogen species. While concrete evidence is still lacking, it has been suggested that these species are involved in neurodegenerative disorders such as Parkinson's [48] and Alzheimer's [49] disease, several kidney diseases [50], as well as chronic inflammatory diseases such as rheumatoid arthritis [51]. Specifically, peroxynitrite-mediated reactions with amino acid residues result in the formation of nitrotyrosine, which can lead to enzyme inactivation [33, 52].

3.4. Specific Cellular Damage

Precise cellular damage resulting from RONS is specifically related to which macromolecules are being targeted by the oxidants, the frequency and duration of attack, as well as the tissue-specific antioxidant defenses present. For example, polyunsaturated fatty acids comprising lipid membranes can be degraded through the chain reaction sequence of lipid peroxidation. Proteins can undergo oxidation leading to modifications in enzyme activity, altering normal cellular functioning. Radicals reacting with DNA can produce extensive strand breakage and degradation of deoxyribose, an effect that has been shown *in vitro* to be due to the formation of hydroxyl radicals [43]. Such changes leading to alterations in nucleotide bases certainly have the potential to be mutagenic, perhaps promoting ill-health and disease over time. Any of the above, either alone or in combination with one another, can lead to impaired health and physiological dysfunction, potentially impairing physical performance.

3.4.1. *Methods of Assessing RONS Formation*

3.4.1.1. *Direct Methods.* Since radicals are highly reactive and short lived (e.g., 10^{-6}, 10^{-5}, 10^{-9} s for singlet oxygen, superoxide radical, and hydroxyl radical, respectively) they are extremely difficult to measure in biological systems, in particular, plasma and other body fluids. While this is true, there do exist direct procedures for measuring free radical activity, most common being electron spin resonance (ESR) spectroscopy involving spin traps (which allows for a more stable product), in addition to less common techniques such as pulse radiolysis and laser flash photolysis [33]. The equipment needed for analysis of samples using these techniques is costly, and the procedures are complex and labor intensive, making the analysis of large batches of samples difficult. Therefore, few studies have employed these techniques to study radical formation following an acute bout of exercise [53–57], with increased radical formation detected using ESR first demonstrated in muscle and liver tissue following exhaustive exercise in animals in 1982 [56] and several years later in humans [53].

3.4.1.2. *Indirect Methods.* The majority of investigations focused on exercise-induced oxidative stress have used indirect methods as a way to determine changes in tissue oxidation of lipids, proteins, and DNA resulting from exposure to RONS. In addition, alterations in components of the endogenous antioxidant defense system, in particular glutathione status (e.g., increased oxidized and decreased reduced glutathione) and water- and lipid-soluble vitamins, have been used as markers of oxidative stress. Using this approach, radical formation is inferred based on the nature of oxidation caused to biological molecules, as well as the decrease in antioxidant capacity. Related to the latter, although not as routinely measured as lipid, protein, and DNA oxidation, a decrease in circulating levels of antioxidant vitamins (C and/or E) following acute exercise [58] and short-term overreaching [20] has been noted, with no change [59] and an increase noted in other investigations involving acute exercise [60, 61].

A variety of analysis procedures have been used [62], ranging from simple spectrophotometric assays to more complex and time-consuming assays using gas chromatography–mass spectroscopy (GC–MS) and high-performance liquid chromatography (HPLC) coupled with electrochemical or chemiluminescence detection. Procedures are available for analysis of several body fluids (e.g., blood, urine, saliva), as well as muscle and organ tissue (which have been widely studied in animal investigations focused on exercise and oxidative stress). In human studies of exercise and oxidative stress, blood and urine have been the analysis tissue of choice in most investigations.

3.4.2. *Common Biomarkers of Exercise-Induced Oxidative Stress*

Because of the increased interest in the study of oxidative stress in general, and exercise-induced oxidative stress in particular, several commercially available assay kits are now available, with many new kits emerging each year. In fact, while many companies make available oxidative stress-related reagents and assay kits as one component of the entire product line, other companies now offer oxidative stress-related reagents and assay kits exclusively, at the same time also providing analytical services for these procedures. Clearly, this area of study is on the rise, perhaps partly because the analytical tools needed for this work are more user friendly and readily available than ever before. Table 2 presents an overview of common biomarkers of exercise-induced oxidative stress, as well as the format of analysis for each biomarker.

3.4.3. *Common Targets and Consequences*

As previously mentioned, an elevation in RONS may promote oxidation of a variety of molecules, potentially leading to impaired health and physical performance. Lipids have received the greatest deal of attention in relation to

TABLE 2
COMMON BIOMARKERS OF EXERCISE-INDUCED OXIDATIVE STRESS

Molecule	Biomarker	Common assessment format(s)
Lipid	Isoprostane	GC–MS ELISA
	Lipid hydroperoxides	Spectrophotometric ELISA
	Malondialdehyde	HPLC Spectrophotometric
	Thiobarbituric acid reactive substances	HPLC Fluorometric Spectrophotometric
	Conjugated dienes	Spectrophotometric
	Oxidized low-density lipoprotein	*Ex vivo* oxidation followed by lag phase of conjugated diene formation ELISA
Protein	Protein carbonyls	Spectrophotometric ELISA Western blot
	Individual oxidized amino acids	GC–MS Western blot
	Nitrotyrosine	ELISA
DNA	8-Hydroxydeoxyguanosine	HPLC ELISA
	Oxidized DNA bases	HPLC
	Strand breaks	Comet assay
Antioxidant[a]	Glutathione	HPLC Spectrophotometric
	Trolox equivalent antioxidant capacity	Spectrophotometric
	Ferric-reducing ability of plasma	Spectrophotometric
	Total radical-trapping antioxidant parameter	Spectrophotometric
	Oxygen radical absorbance capacity	Fluorometric
	Glutathione peroxidase	Spectrophotometric
	Glutathione reductase	Spectrophotometric
	Superoxide dismutase	Spectrophotometric
	Catalase	Spectrophotometric
	Ascorbate (vitamin C)	Spectrophotometric
	Tocopherol (vitamin E)	HPLC
Miscellaneous	Xanthine oxidase	Fluorometric Spectrophotometric
	Hydrogen peroxide	Fluorometric Spectrophotometric

ELISA = enzyme-linked immunosorbent assay; GC–MS = gas chromatography–mass spectroscopy; HPLC = high-performance liquid chromatography.

[a] Multiple other individual antioxidants can be measured; however, this has been done rarely in the exercise science literature.

an acute bout of exercise. The autocatalytic process of lipid peroxidation is linked to increased mitochondrial respiration and electron transport disturbances associated with increased oxygen uptake (VO_2) during exercise and involves degradation of polyunsaturated fatty acids and phospholipids through a chain reaction [63]. This includes removal of a hydrogen atom from one of the $–CH_2–$ groups in the carbon chain, typically initiated by a hydroxyl radical, leaving behind an unpaired electron on the carbon. The resulting carbon radical undergoes molecular rearrangement to form a conjugated diene, which can then combine with oxygen to give rise to a peroxyl radical. Peroxyl radicals are capable of abstracting a hydrogen atom from other fatty acids and giving rise to a chain reaction forming lipid hydroperoxides in cell membranes, tissues, and body fluids (Fig. 2).

These reactions are not limited only to the site of free radical generation, but can also occur at sites far removed from the initial location due to the

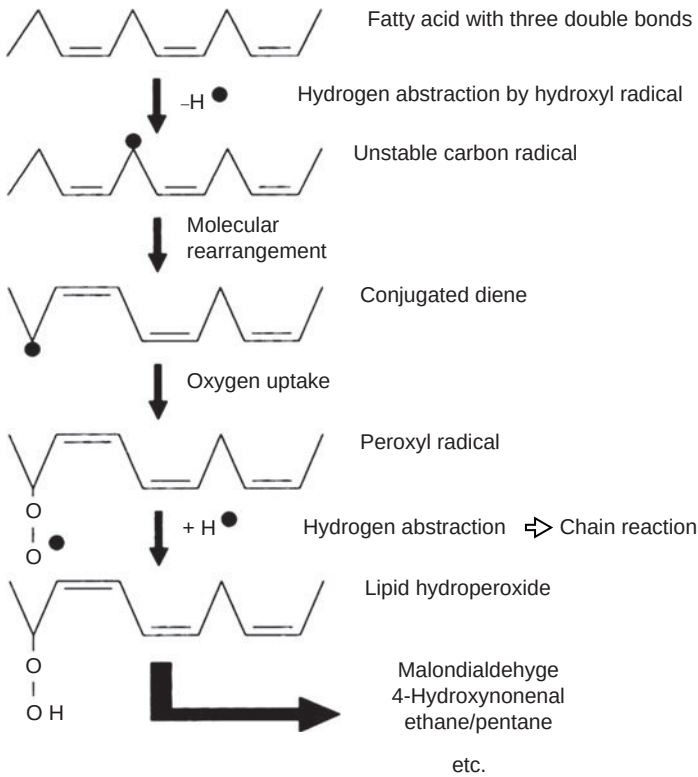

FIG. 2. Phases of lipid peroxidation. Used with permission from Young and McEnemy [231].

ability of peroxyl radicals to travel through the bloodstream. Here, a single initial free radical reaction can result in the formation of numerous lipoperoxides until all of the membrane fatty acids are completely oxidized to hydroperoxides or the reaction is terminated either by an antioxidant chain breaker or by two free radicals meeting and nullifying each other. Lipid peroxidation changes cell membrane fluidity and increases membrane permeability, often represented by the leakage of various intracellular proteins into the circulation (e.g., creatine kinase, lactate dehydrogenase).

Three phases of lipid peroxidation have been described and are as follows: initiation, propagation, and termination [64]. The initiation phase involves the removal of one or more hydrogen atoms from the polyunsaturated fatty acid, resulting in the formation of conjugated dienes. In the propagation phase, the carbon-centered fatty acid radicals combine with molecular oxygen, yielding the highly reactive peroxyl radicals that are capable of producing new fatty acid radicals, resulting in the radical chain reaction. Here, the peroxyl radicals themselves are converted to stable termination phase products, the lipid hydroperoxides. These lipid hydroperoxides appear stable under physiological conditions until they come into contact with transition metals such as iron and copper, which cause decomposition, producing different reactive molecules that can further promote the chain reaction (Fig. 2).

The overall process of lipid peroxidation may result in the accumulation of a wide variety of by-products, including hydrocarbon gases (e.g., pentane, ethane) and aldehydes. Some of these by-products are thought to accumulate in cells, giving rise to the so-called ''age-related pigment'' or lipofuscin [33]. With extensive damage, normal physiological function may be impaired (i.e., loss of membrane fluidity, increased membrane permeability with loss of cytosolic proteins and alteration in enzyme function), and in extreme cases lead to cell death. Indeed, lipid peroxidation is thought to play an important role in the etiology of many pathological conditions, such as cancer, atherosclerosis, and possibly other degenerative diseases.

There are several approaches to assess lipid peroxidation, including the oxidation susceptibility of body fluids [or isolated lipid fragments such as low-density lipoprotein (LDL)] *in vitro* by using oxidizing agents such as transition metals as initiators of lipid peroxidation, as well as the measurement of oxidation products formed *in vivo*. Assessment of the latter has included the study of conjugated dienes, lipid hydroperoxides (LOOH), thiobarbituric acid reactive substances (TBARS), and an assessment of one of the major aldehydes, the 3-carbon chain malondialdehyde (MDA). Although the assay for TBARS is widely used, it lacks specificity, as it measures aldehyde breakdown products of some (e.g., MDA) but not all lipid hydroperoxides, as well as reacts with saturated and unsaturated

nonfunctional aldehydes. For this reason, measurement of F_2-isoprostanes, a prostaglandin-like compound generated *in vivo* by nonenzymatic peroxidation of arachidonic acid, is now considered a more reliable marker of lipid peroxidation.

While it is well accepted that RONS promote oxidation of lipids following acute exercise, far less attention has focused on RONS-mediated protein oxidation. This may be because proteins undergo constant turnover within the human body, and any damage due to RONS would not result in any significant accumulation of modified protein structures. However, more attention has been given recently to the study of proteins in relation to exercise-induced oxidative stress, with investigations demonstrating significant oxidative damage to proteins, often measured by the presence of carbonyl derivatives.

RONS can react directly with proteins or they can generate secondary products, which may react with proteins, by first reacting with molecules such as lipids or sugars. When exposed to oxidant species in the presence of transition metal ions, either the protein side chain or the peptide bond may be oxidized. Aromatic and sulfhydryl-containing residues are particularly susceptible to oxidation [65]. Specifically, cysteine residues are prone to a range of oxidative modifications, including *S*-glutathiolation [66]. Reactions may involve specific alterations such as the conversion of phenylalanine residues to *o*-tyrosine and of tyrosine to dityrosine, or more global modifications resulting in the formation of carbonyl derivatives. Such changes may lead to loss of catalytic, contractile, or structural function in the affected proteins and making them susceptible to proteolytic degradation [67]. Further, the susceptibility to oxidation may be protein specific, such that certain proteins are more likely to undergo oxidation than others. In the exercise science literature, protein carbonyl content is the most widely used marker of oxidative modification to proteins, perhaps because it appears to provide an overall assessment of the burden borne on the cell as a consequence of oxidative stress.

Oxidation of DNA usually involves damage to single bases, with different modifications observed depending on the RONS interacting with the DNA. It has been proposed that there may be, on average, 10^3 oxidant-mediated damaging events upon the DNA of each cell in the human body per day [32]. Damage may occur to both mitochondrial and nuclear DNA, and may involve DNA strand breaks as well as oxidative base modifications. In the study of exercise-induced oxidative stress, the most commonly used technique in recent years is the measurement of 8-hydroxy-2′-deoxyguanosine (8-OHdG) formation, which has been assessed in muscle and organ tissue, urine, serum, and isolated leukocytes. Because 8-OHdG is not a normal intermediate in nucleotide metabolism, its presence is used to indicate

oxidative DNA damage. While only a small percentage (~10%) of total oxidative DNA damage is represented by 8-OHdG, it has a high potential for mutagenesis, is frequently found in tumor-related genes, and has elevated concentrations associated with various physiological diseases and disorders, in particular, both aging and cancer [68].

In relation to the above discussion, it should be remembered that when assessing and evaluating biological systems, any particular assay procedure is merely capturing a "snapshot" of what is occurring at that particular time. That is, it is quite possible that when taking a single sample following an acute bout of exercise, the generation of RONS and associated oxidation to macromolecules could be "missed," either by taking the sample too late or by not waiting long enough for secondary generation of RONS and associated oxidation (assuming this is the case). Therefore, it is best to take repeated samples following a bout of exercise, possibly for several hours or days into recovery when the exercise is extreme and believed to induce muscle injury. Such a time course analysis allows for better representation of the oxidative status of the system.

Further, as clearly expressed in recent reviews on the subject [2, 69–71], no one assay can accurately represent the entire process of oxidation within cells. This is underscored by investigations demonstrating an increase in certain biomarkers, no changes in others, and sometimes a decrease in others. As such, it is best to include assays specific to oxidation of several macromolecules (e.g., protein, lipids, DNA). In this way, a better understanding of the overall oxidative stress can be obtained, as the status of some macromolecules may be altered while others may not be.

3.5. Protective Mechanisms Against RONS

While RONS are constantly generated in cells and increase with physical exertion, their production does not necessarily lead to cellular modification and degradation. This is because there exist numerous defenses either to minimize RONS formation or to neutralize their damaging effects once formed. These may be categorized as follows: antioxidant enzymes, antioxidant scavengers and miscellaneous antioxidant compounds, and metal-binding proteins. Some of the most prevalent and well described are presented in the following sections and in Table 3.

3.5.1. *Antioxidant Enzymes*

The discovery of SOD by McCord and Fridovich [72] fueled the acceptance that free radicals are important in biological systems. In regards to SOD, three primary forms are known to exist: a cytosolic copper-zinc enzyme (Cu-ZnSOD), a mitochondrial enzyme requiring manganese (MnSOD), and

TABLE 3
ANTIOXIDANT DEFENSE MECHANISMS

Major antioxidant enzymes	Nonenzymatic antioxidant	Metal-binding proteins
Superoxide dismutase	Vitamin A (retinol)	Hemoglobin
Cu-ZnSOD	Vitamin C (ascorbate)	Myoglobin
MnSOD	Vitamin E (tocopherol)	Ceruloplasmin
Extracellular SOD	Carnitine	Ferritin
Glutathione peroxidase (selenium)	Coenzyme Q_{10}	Lactoferrinact
Catalase	Thiols	Metallotheionein
Glutathione reductase	Uric acid	Transferrin
Glutathione S-transferase	Bilirubin	
	Carotenoids (beta-carotene, lycopene, etc.)	
	Flavonoids (quercetin, catechin, etc.)	
	Lipoic acid	
	N-Acetyl-cysteine	
	Resveratrol	
	Selenium	

an extracellular SOD (EC-SOD). These enzymes act to catalyze the conversion of superoxide to hydrogen peroxide as follows:

$$O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2$$

As mentioned previously, while hydrogen peroxide itself is not considered a radical, nor is it highly toxic, when in the presence of various transition metal ions such as copper and iron, it has the ability to convert to the highly reactive hydroxyl radical (via the Fenton reaction).

The enzymes glutathione peroxidase (GPx) and catalase (CAT) function to inactivate much of the hydrogen peroxide before reacting with the transition metals in both the cytosol and in intracellular organelles (i.e., peroxisomes), respectively. Specifically, GPx, a selenium-dependent and selenium-independent enzyme, in the presence of GSH catalyzes the conversion of both organic peroxides and hydrogen peroxide to water and GSSG as follows:

$$ROOH + 2GSH \rightarrow GSSG + ROO$$

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$$

In a similar manner, CAT converts hydrogen peroxide to water and oxygen as follows:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

In relation to GPx and the formation of GSSG, the enzyme glutathione reductase (GR) catalyzes the reaction to regenerate GSH using NADPH as the hydrogen donor as follows:

$$\mathrm{GSSG} + 2\mathrm{NADPH} \rightarrow 2\mathrm{GSH} + 2\mathrm{NADP}^{+}$$

Here, the production of NADPH is coupled with glucose-6-phosphate dehydrogenase (G6PDH) as follows:

$$\mathrm{Glucose}-6-\mathrm{phosphate} + \mathrm{NADP}^{+} \rightarrow 6\ \mathrm{Phosphogluconate} + \mathrm{NADPH}$$

The enzyme glutathione S-transferase (GST) acts to catalyze the reaction between the –SH group of GSH and possible alkylating agents, allowing GSH to carry out its detoxifying functions.

3.5.2. *Nonenzymatic Antioxidants*

Aside from antioxidant enzymes that provide protection against RONS attack, humans are equipped with various natural free radical scavengers/chain breakers. Most notably may be the class of organic sulfur derivatives known as thiols, which have numerous functions, including a pivotal role in coordinating the antioxidant defense system by acting as reducing agents [73].

Glutathione is the major nonenzymatic nonprotein thiol in cellular systems, representing approximately 50% of total thiols [73] and typically existing primarily in the reduced form (GSH). However, when donating its hydrogen, as in the protective conversion of hydrogen peroxide to water, GSH can be transformed into oxidized glutathione (GSSG). This oxidized form of glutathione can be reduced back to GSH by the action of glutathione reductase in the presence of NADPH or NADH, or may be released into extracellular compartments.

Investigators have routinely measured whole blood glutathione concentrations as a marker of oxidative stress within biological systems, as this appears one of the most reliable indices of exercise-induced oxidant production [74]. Since less than 1% of total blood glutathione (TGSH) is found in plasma [75], glutathione status is often assessed in whole blood. This can be accomplished by measuring separately, both TGSH and GSSG, and mathematically computing GSH.

In addition, dietary intake supplies further antioxidants in the form of vitamins (e.g., A, C, E), minerals (e.g., selenium, zinc), carotenoids (e.g., beta-carotene), flavonoids, and phenols. The vast supply of antioxidants in the form of dietary constituents remains to be elucidated, as does the precise amount necessary to yield optimal health and physiological functioning. This is especially true for exercise-trained individuals, as their own endogenous

antioxidant defense mechanisms are generally upregulated as a training adaptation [76]. Despite upregulation of the defense system, the body's antioxidant capacity is not sufficient to totally prevent exercise-induced oxidative stress. Therefore, it is possible that even elite athletes could benefit from antioxidant supplementation, a question that remains open for debate.

Many of these antioxidants function together to provide cellular protection *in vivo*. For example, during lipid peroxidation, tocopherol (vitamin E) functions as a potent chain-breaking antioxidant, intercepting lipid peroxyl radicals ($LOO^{\bullet}$), and forming a vitamin E radical as a product. Ascorbate (vitamin C) acts to regenerate vitamin E by accepting the electron from the vitamin E radical, with the vitamin C radical (dehydroascorbate) being formed, and either excreted in the urine or regenerated to vitamin C via electron donation from GSH (Fig. 3). It should be noted that both vitamin E and C function to scavenge superoxide radical and hydroxyl radical in the lipid and aqueous phases, respectively, inhibiting lipid peroxidation and oxidative damage to other macromolecules.

In addition to the protection that is afforded through antioxidant enzymes and scavengers, other important natural mechanisms are available to aid in the prevention of free radical formation. Certain minerals such as selenium, copper, and zinc play important roles in the support of other antioxidant systems, specifically, GPx and Cu-ZnSOD, respectively. The vitamin A precursor beta-carotene has important properties as an antioxidant, functioning mainly to suppress singlet oxygen. Various flavonoids can function as quenchers of singlet oxygen, hydrogen donators (i.e., chain-breaking antioxidants), and reducing agents. In addition, metal-binding proteins that are bound to transition metals, preventing them from reacting with peroxides to generate radicals via both Fenton and Haber-Weiss reactions, can serve as antioxidant agents, perhaps indirectly. The antioxidant pathways may be complimented by the synthesis of heat shock proteins, stress proteins that function to repair and maintain protein folding [77]. In the above ways, antioxidant agents help to support physiological functioning by either reducing the amount of free radical formation or by attenuating the progression of RONS attack and damage to cell structures.

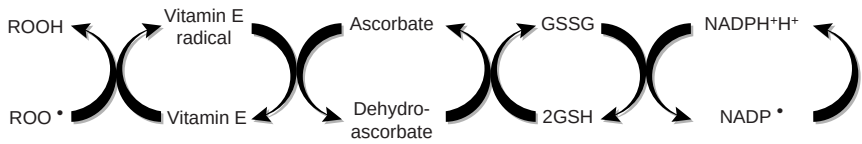

FIG. 3. Interaction of antioxidants in redox cycling.

3.5.3. *Exercise-Induced Alterations in Antioxidant Defense*

Some health benefits of regular exercise stem from upregulation of antioxidant defenses, likely coupled with decreased RONS formation [76, 78–80], which is often associated with decreased oxidative stress at rest and following acute exercise. For adaptations to occur, exercise needs to be of high enough volume and intensity to cause acute elevations in RONS production, as detailed earlier [2, 45]. In other words, a "sublethal" amount of oxidative stress is needed for such positive adaptations. Seemingly, the adaptations result from the cumulative effect of repeated exercise bouts and exposure to RONS. It should be mentioned that although antioxidant defenses may be increased as a result of chronic exercise training, oxidative stress following acute bouts of submaximal exercise is typically not eliminated, but rather reduced.

For specific information related to changes in the endogenous antioxidant defense system resulting from chronic exercise training, the reader is referred to the several excellent reviews pertaining to this topic [76, 78–80]. Although more studies have focused on adaptations resulting from aerobic exercise, evidence exists following both aerobic and anaerobic training interventions. The adaptations are not limited to blood and skeletal muscle, but also often apply to organ tissue. In fact, a significant emphasis has been placed on exercise-induced cardioprotection [80], which appears associated with enhanced antioxidant defense and decreased RONS formation.

As with biomarkers of oxidative stress, there exist several biomarkers/assays representing antioxidant status. These include the common enzymatic and nonenzymatic antioxidants as presented in Table 3, which are often measured in a variety of tissues and blood. In addition, because of the wide array of antioxidant components present in blood and the difficulty in measuring each component separately, several methods have been developed to assess the "total" antioxidant capacity of blood. These include the Trolox equivalent antioxidant capacity (TEAC) assay (which is primarily influenced by urate), the oxygen radical absorbance capacity (ORAC) assay, the ferric-reducing ability of plasma (FRAP) assay, and the total radical-trapping antioxidant parameter (TRAP) assay. Of these, ORAC and FRAP appear to correlate well, although TEAC does not correlate with either ORAC or FRAP, and may underestimate antioxidant capacity [81]. Therefore, observations derived from these measurements should be viewed with caution.

While these assays have been used in the exercise science field and have been extensively evaluated, there is no one assay considered to be "ideal" at the present time [82] and hence, no uniform use of these assays exists across studies. The majority of investigations have noted an increase in blood antioxidant status using these assays, although the magnitude of increase has varied across studies. Discrepancies may be largely due to the use of

different assay procedures and the difficulty in comparing results across studies in which different procedures have been used. Therefore, as with the biomarkers of oxidative stress, it has suggested that multiple assays of antioxidant status be included to best characterize the system [83].

4. Exercise and Oxidative Stress

Based on evidence presented in more than 300 original investigations over the past 30 years, it is clear that exercise of sufficient intensity and duration increases the formation of RONS, creating an imbalance between oxidant and antioxidant levels, which promotes an increase in oxidative stress biomarkers. Specifically, oxidative stress increases in both an intensity- and duration-dependent manner.

In one of the early studies on exercise-induced oxidative stress, Lovlin *et al.* [84] reported an increase in MDA in subjects immediately following a graded exercise test to exhaustion, while exercise at 70% of maximal oxygen consumption (VO_{2max}) and lower percentages demonstrated no increase in this biomarker. Leaf *et al.* [85] measured expired ethane and pentane as markers of lipid peroxidation while subjects performed a graded exercise test to exhaustion. While values were elevated above rest at approximately 60%VO_{2max}, they continued to rise and were greatest at the end of the test, indicating an intensity-dependent response. More recent evidence supports these findings in that exercise intensity plays a major role in postexercise oxidative stress [86]. Collectively, these data demonstrate an intensity-dependent response, at least in regards to lipid peroxidation induced by primarily aerobic exercise.

In support of the data in exercising humans, animal studies have shown that while forced exercise can significantly increase the level of macromolecule oxidation [87], likely due to the maintenance of high-intensity work, voluntary exercise performed at low intensities results in either no change or only a minimal effect on oxidative stress biomarkers [88]. These findings reinforce the idea that exercise needs to be of sufficient intensity to result in accumulation of RONS and subsequent oxidative stress. However, in opposition to the above findings, one recent study employing resistance exercise (squats) noted an increase in MDA, which occurred independent of exercise intensity [89]. It is likely that the oxidative stress response to exercise is mode dependent, as reported earlier [90].

As for exercise duration, only one study to date [91] has directly compared oxidative stress (measured by protein carbonyls) in response to different exercise durations (i.e., 30, 60, and 120 min). In this investigation it was shown that protein oxidation increased following steady state, fixed load cycling exercise, and did so as a function of exercise duration (Fig. 4).

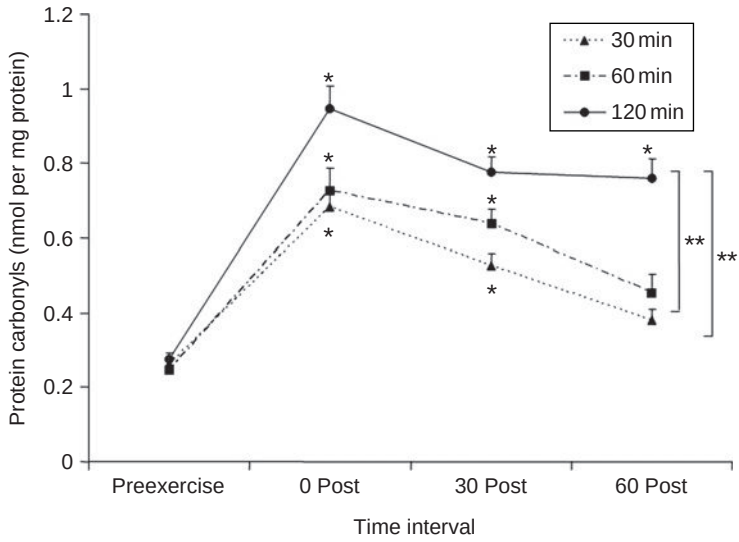

FIG. 4. Plasma protein carbonyls collapsed over eight men and seven women before and following 30, 60, and 120 min of cycling at 70%$VO_{2\ peak}$ (mean ± SEM). * Protein carbonyl concentration greater than pre-exercise ($p < 0.05$). ** Protein carbonyl concentration greater at all times postexercise for the 120-min condition compared to the 30- and 60-min conditions ($p < 0.05$); no difference between the 30- and 60-min conditions at any time postexercise ($p > 0.05$). Used with permission from Bloomer *et al.* [91].

Moreover, the response was similar for both men and women. Other studies measuring oxidative stress following marathon and ultramarathon competitions have shown elevations in certain biomarkers that appear to exceed the rise in similar biomarkers following shorter duration (e.g., 30–60 min) exercise bouts. For example, both Okamura *et al.* [92] and Poulsen *et al.* [93] reported that excessive aerobic exercise (30 ± 3 km/day for 8 days and 10 hours/day for 30 days, respectively) significantly increased urinary 8-OHdG levels during the training period to a greater extent than is observed following shorter duration exercise bouts.

While not all investigations have reported an increased oxidative stress following acute exercise, most have noted an increase in certain biomarkers during the acute (minutes to hours) postexercise period, with some exercise bouts (e.g., extreme duration and intensity aerobic, resistance exercise involving muscle injury) leading to longer lasting changes in oxidative stress biomarkers. The extent of oxidation of these molecules varies across studies and is likely due to factors such as the type, intensity, volume, and duration of exercise; the exercise training and nutritional status of the research subjects;

the time course of sample collection; the tissue being investigated (e.g., blood, urine, muscle); and the various assays employed as part of the research design.

Another consideration related to exercise-induced oxidative stress is the use of antioxidant supplements, which has resulted in mixed findings in both humans and animals. The reader is referred to the following review articles on this topic as pertaining to both aerobic [94–96] and anaerobic [97] exercise for further information. Although several antioxidants have been used within research designs, those that are most prevalent are vitamins C and E. Potential explanations for the mixed findings include differences in the type, dosage, timing, and route of administration of the antioxidants; the training status of the research subjects/animals (e.g., exercise trained vs untrained); as well as the baseline antioxidant status of the research subjects/animals. While antioxidant pretreatment has shown to be beneficial at attenuating exercise-induced oxidative stress in several investigations, more work is needed in this area before specific recommendations can be made with any degree of confidence regarding precise antioxidant treatment regimens for this purpose. Moreover, due to the important role of RONS in regulating several aspects of physiology, it should be questioned as to whether attenuation/elimination of the oxidative stress response to exercise is beneficial. That is, it is likely that the acute rise in oxidative stress following exercise is an important biological signal to allow for upregulation in endogenous antioxidant defense [79].

It should be noted that while numerous studies have indeed shown elevations in oxidative stress biomarkers following acute bouts of exercise, due to the complexity of the exercise (e.g., involvement of multiple organ systems as well as skeletal muscle), changes observed in such biomarkers may reflect RONS formation in any number of tissues, and likely represent a variety of mechanisms acting synergistically to produce RONS. Similarly, the generation of RONS in these systems/tissues may be associated with several factors including increased oxygen flux through the mitochondrial electron transport chain, mechanical stresses, ischemia-reperfusion conditions (in particular in inactive skeletal muscle and organ tissue, which are deprived of blood flow during strenuous exercise), changes in blood-borne variables, in addition to other factors discussed in Section 3.3.1. Indeed, aerobic and anaerobic exercise modes rely on different metabolic pathways for ATP production, and have the ability to induce multiple distinct changes within biological systems. Moreover, the metabolic and mechanical stresses involved in aerobic and anaerobic exercise of differing mode, intensity, volume, duration, and contraction type (e.g., eccentric vs concentric) may dictate alterations in oxidative status. Therefore, the oxidative stress response to these distinct forms of exercise is likely variable. To this author's knowledge, only one

study has compared oxidative stress following these two forms of exercise matched for exercise time using a crossover design [90], and noted a similar or greater increase in oxidative stress biomarkers following anaerobic compared to aerobic exercise. Another recent investigation matched aerobic exercise with climbing (intermittent isometric exercise) performed at the same percentage of VO_{2max} and noted greater oxidative stress following climbing exercise [98]. A few studies have compared aerobic and anaerobic exercise modes in humans [99–100] or animals [101] performing one mode or the other and noted similar changes in oxidative stress biomarkers in humans, and a greater increase in animals following anaerobic exercise. These studies suggest that anaerobic exercise can induce oxidative stress to an extent that parallels, if not exceeds, that seen with aerobic exercise. The relative paucity of data in relation to direct comparisons of exercise modes, in particular matched for duration, intensity, and muscle mass recruited, merits further attention. As mentioned earlier, with few exceptions, human studies of oxidative stress following exercise have measured biomarkers in blood and urine exclusively. In these studies, it is unknown whether changes in skeletal muscle or organ tissue oxidative stress correlate. Therefore, failure to note increased blood or urine oxidative stress does not necessarily indicate that oxidative stress did not occur elsewhere.

One final aspect that deserves consideration is RONS generation when participating in outdoor exercise events, during which time exposure to certain air pollutants may exacerbate RONS formation and hence exercise-induced oxidative stress [102], as increased ventilation carries more pollutants into the respiratory tract. The following text discusses both aerobic and anaerobic exercise bouts in relation to oxidative stress biomarkers.

4.1. Aerobic Exercise

While participation in regular aerobic exercise (e.g., walking, running, cycling) has been shown to be beneficial at reducing both morbidity and mortality rates, it is also known that aerobic exercise of sufficient intensity (i.e., generally $>70\%VO_{2max}$) and duration (i.e., >30 min) can impose a state of oxidative stress. The production of RONS during and following aerobic exercise may be primarily associated with the increase in oxygen uptake, although other pathways as described in Fig. 1 are certainly involved [4], in particular when the exercise involves high-force downhill running, as this activity may induce a significant amount of muscle injury.

Several excellent reviews have been published in reference to aerobic exercise-induced oxidative stress [45, 69, 71, 103]. As with anaerobic exercise, as discussed in Section 4.2, studies have included a variety of exercise modes, intensities, and durations. Some protocols have been performed in the

laboratory, while others have involved outdoor races (i.e., field studies) such as marathons, ultramarathons, triathlons, and cycling events. The following sections provide a general overview of the findings in regard to aerobic exercise-induced oxidative stress.

4.1.1. *Moderate Duration Laboratory Studies: Downhill Running Protocols*

Although running exercise is considered to be aerobic, the use of high-force eccentric muscle actions, as necessary during downhill running, may involve RONS generation mediated through muscle injury pathways such as trauma, inflammation, and a disruption in calcium homeostasis. Therefore, the oxidative stress experienced with these protocols has the potential to persist for hours to days following the exercise bout [104]. Lipid peroxidation has been the target of most studies in this area involving humans. However, animal studies have also noted increased protein oxidation as measured by carbonyls [105], and DNA oxidation as measured by 8-hydroxydeoxyguanosine in white blood cells [106] following downhill running protocols.

In one early human investigation, Maughan *et al.* [104] found that TBARS was elevated following 45 min of downhill running, with a peak value observed at 6 hours postexercise and a return to baseline by 72 hours postexercise. Meydani *et al.* [107] noted increased urinary TBARS adducts for up to 12 days following a 45-min downhill treadmill run. Other work has demonstrated a long-lived (i.e., 24–72 hours) elevation in isoprostanes in both young (26 ± 3 years) and old (71 ± 4 years) subjects following downhill running [108]. More recently, it was reported that lipid hydroperoxides were increased immediately following downhill running, with values returning to baseline at the 24-hour measurement [109]. Other work opposes these findings, indicating no change in MDA or conjugated dienes [110] or GSSG [111] following downhill running. However, despite no change in GSSG in the study by Camus *et al.* [111], ascorbic acid was decreased significantly and myeloperoxidase (a marker of polymorphonuclear neutrophil activity) was increased following the exercise. It is possible that the oxidant stress associated with some protocols is insufficient to alter certain oxidative stress biomarkers. It also needs to be considered that differences in blood, skeletal muscle, and organ tissue oxidative stress may be present [105].

4.1.2. *Moderate Duration Laboratory Studies: Noneccentric Biased Protocols*

Unlike downhill running, laboratory protocols using traditional walking, jogging, and cycling exercise do not involve a great deal of high-force eccentric muscle actions and are not thought to cause any significant amount

of muscle injury. Investigations in this area are numerous and have generally resulted in increased lipid [112–118], protein [91, 119–123], and glutathione [90, 117, 121, 124–130] oxidation in a variety of tissues (e.g., skeletal muscle, cardiac, liver), as well as in both blood and urine. However, with few exceptions [115, 122], studies have failed to note an increase in DNA oxidation following moderate duration aerobic exercise [59, 130–134]. It is possible that longer duration, higher intensity exercise is needed to promote DNA oxidation, as shown in Section 4.1.3.

The findings of increased oxidative stress following moderate duration aerobic exercise bouts are well documented for humans, as indicated above, as well as for animals involved in treadmill walking/running [135–147] and swimming [118, 148–149]. In much of the animal work, investigators have noted increased MDA [118, 139] and TBARS [150], protein carbonyls [105] and 8-hydroxydeoxyguanosine [151] in skeletal muscle [105, 118, 147, 151], heart [118], liver [139], and kidney [150].

In regards to moderate duration aerobic exercise studies, the oxidative stress response in most cases is transient, and values return toward baseline within minutes following exercise in most investigations. A recent study highlights this nicely, reporting that several oxidative stress biomarkers are altered during the 20 min following graded exercise, but return to basal values by 30-min postexercise [152]. Our data for protein carbonyls following different exercise durations, as presented in Fig. 4, show this trend [91]. We have noted similar findings for MDA and glutathione status following 30 min of aerobic cycling [90].

Taken together, the data indicate that oxidative stress as measured by lipid, protein, and glutathione oxidation can increase following moderate duration aerobic exercise, with a rapid return toward basal levels following the cessation of the exercise stimulus. The short-lived rise may be due to increased catabolism, excretion, or redistribution throughout the body, in addition to activation of redox reactions (e.g., conversion of oxidized to reduced glutathione via glutathione reductase).

4.1.3. *Long Duration Field Studies*

As with other forms of aerobic exercise, the performance of single bouts of long duration trials, such as marathon running (26.2 miles), distance cycling, triathlons, and other long duration sporting events, has resulted in increased oxidative stress. This has been demonstrated repeatedly for lipids [92, 153–158], DNA [92–93, 159–161], and glutathione [162]. The time course of such increases in oxidative stress biomarkers extends beyond the acute postexercise period, as reports of increased oxidized macromolecules exist for periods through 1 week of recovery [161]. These findings are interesting in that despite subjects' training status and ability to perform relatively high-intensity

exercise bouts for long periods of time, signs of oxidative stress are still evident. This suggests that regardless of their high level of training, likely heightened endogenous antioxidant defense system of subjects is not adequate to provide complete protection against exercise-induced oxidation. In contrast to the above findings, a few studies have noted no significant change in oxidative stress biomarkers following long duration aerobic exercise events [163–165], with some authors reporting a slight decrease from pre- to postexercise [166–167].

4.1.4. *Summary of Aerobic Exercise*

As outlined above, several forms of aerobic exercise can induce an acute state of oxidative stress, as evidenced by increased biomarkers of lipid, protein, DNA, and glutathione oxidation in a variety of tissues and body fluids. When the exercise session includes exaggerated eccentric muscle actions and/or involves excessive duration, the oxidative stress can persist for several hours to days following exercise cessation. It is likely that further RONS production in response to localized or systemic injury and stress may be responsible for the longer lived oxidative stress observed in these conditions.

4.2. Anaerobic Exercise

As with aerobic exercise training, the performance of anaerobic exercise (e.g., resistance training, sprinting, jumping), an activity that does not require oxygen to fuel muscular work, is associated with multiple health benefits including favorable effects on muscular strength and endurance, body composition, cardiovascular functioning, metabolism, and psychological well-being [168]. However, as with the performance of acute bouts of aerobic training, this form of exercise has also been shown to increase oxidative stress, as reviewed in detail previously [2]. The production of RONS during and following anaerobic exercise is possible through a number of pathways as described in Fig. 1: in addition to a potential depletion of glycolytic substrates, an accumulation of substrates that generates oxidants, and a decrease in endogenous antioxidant defense. Unlike aerobic exercise studies for which more than 250 published articles exist, studies related to anaerobic exercise-induced oxidative stress are far less. Therefore, a more comprehensive overview is possible here.

4.2.1. *Dynamic (Eccentric/Concentric) Resistance Exercise*

Dynamic resistance exercise involving both concentric (shortening) and eccentric (lengthening) muscle actions is the most commonly performed type of anaerobic exercise by the population at large. This type of exercise

primarily utilizes free weight barbells/dumbbells and weight machines. While concentric muscle actions require a greater energy cost compared to eccentric actions [169], eccentric actions are principally responsible for inducing muscle injury, as described earlier in detail [170]. Performed together, concentric and eccentric muscle actions can generate RONS via several pathways, as described in Fig. 1.

While more work has been done in recent years investigating oxidative stress resulting from acute bouts of dynamic resistance exercise, only a small number of studies currently exist in the literature, and many have measured lipid peroxidation exclusively. McBride *et al.* [171] were the first to demonstrate that a full-body protocol of dynamic resistance exercise increases oxidative stress during the postexercise period, as measured by blood MDA. Several other investigators have reported increased lipid peroxidation following an acute bout of dynamic resistance exercise [55, 89, 172–175]. Others have reported no change in lipid peroxidation following dynamic resistance exercise [59, 90, 120, 155, 176].

A few investigators have measured protein oxidation following dynamic resistance exercise in both blood [59, 90, 120] and skeletal muscle [177]. In all of these studies, protein carbonyls were used as the biomarker of oxidative stress. Bloomer *et al.* [90] first reported that protein carbonyls are increased following dynamic resistance exercise. These investigators noted a rise in protein carbonyls immediately postexercise, with a more robust rise observed at 24-hours postexercise. These data indicate that RONS production may have been further increased at times several hours postexercise, possibly mediated by alterations in calcium homeostasis, as well as increased neutrophilia [86, 111], both of which are contributors to RONS production and which coincide with muscle injury. In support of this hypothesis, subjects reported extreme muscle soreness during the 24-hour period following the exercise protocol (30 min of dumbbell squats using 70% of one-repetition maximum), a sign suggestive of muscle injury.

Aside from lipid and protein oxidation, DNA oxidation has been measured following dynamic resistance exercise in two investigations [90, 120]. In both of these studies, no change in DNA oxidation, as measured by serum 8-hydroxydeoxyguanosine, was noted. It is possible that DNA is better protected against oxidative stress than lipids and proteins, perhaps due to the ability of DNA to undergo rapid repair once oxidized. For example, Radak *et al.* [178] reported that a single bout of exercise can increase the activity of human 8-oxoG DNA glycosylase, a DNA repair enzyme that functions to curb the elevation in 8-OHdG. Greater exercise volume and intensity, coupled with more frequent sampling (as opposed to isolated samples taken immediately or 24-hours postexercise), may be necessary to observe significant changes in 8-OHdG following this form of exercise.

Finally, xanthine oxidase activity has been reported to increase in one investigation using dynamic resistance exercise [175]. It is possible that increased xanthine oxidase activity could lead to an increased production of superoxide. Although to this author's knowledge, this has yet to be shown in reference to dynamic resistance exercise.

4.2.2. *Eccentric Resistance Exercise*

Because high-force eccentric muscle actions have the potential to induce significant muscle injury, in particular in subjects who are unaccustomed to such actions [170], several investigators have used eccentric muscle actions exclusively, as a form of anaerobic exercise to induce oxidative stress. The chosen exercise in these studies has often involved single joint movements (e.g., elbow or knee flexion or extension) in which a major burden has been placed on the exercised muscle(s) in an attempt to induce significant trauma. Moreover, in most studies, test subjects have been nonresistance trained (i.e., unconditioned to such extreme muscle activity), making the degree of insult that much more intense. Although few studies have directly compared the oxidative stress response to exercise in trained and untrained subjects, greater lipid peroxidation has been noted in untrained compared to trained men following exercise [114, 173, 179]. It is possible that such findings are related to a greater antioxidant capacity in trained subjects [114].

As described earlier and depicted in Fig. 1, RONS generation can often occur during the hours or days following an acute bout of exercise in which a significant degree of muscle damage has occurred. Here, production of RONS may be related more to inflammatory processes and imbalances in muscle calcium homeostasis than to changes occurring during the acute exercise period.

As with dynamic resistance exercise, only a small number of investigations have been published using an eccentric exercise model to study exercise-induced oxidative stress, with mixed results. Saxton *et al.* [180] were the first to study oxidative stress in relation to isolated eccentric and concentric muscle actions. Subjects performed both eccentric and concentric actions with their elbow flexors, as well as with their knee extensors (using different limbs for the different muscle actions) for study of lipid and protein oxidation. Both blood (following the arm protocols) and muscle (following the leg protocols) samples were taken at rest and for up to 10 and 2 days postexercise, respectively. Only a slight change was noted for both TBARS and conjugated dienes following the arm protocols, with no change noted for MDA in muscle. However, protein carbonyls were increased in muscle immediately following concentric activity, with no change noted for eccentric exercise. These findings suggest that concentric actions, which require a greater amount of energy to perform and which may involve transient

periods of ischemia/reperfusion, can increase protein oxidation in muscle to a greater extent than eccentric actions, which have a relatively low metabolic cost.

Lipid peroxidation biomarkers were increased following eccentric exercise in studies measuring MDA [181], lipid hydroperoxides, and isoprostanes [182]. Other investigators have reported no change in either blood [183–184] or muscle MDA [185] following high-force eccentric muscle actions. It is tempting to suggest that the findings of no significant increase in lipid peroxidation following eccentric exercise in these studies is due to an inefficient training stimulus (i.e., too low volume and intensity) and/or use of well-trained test subjects who are better adapted to handle the insult. However, comparison across studies does not indicate this to be the case. In fact, Lenn *et al.* [184] had subjects perform 50 maximal eccentric actions using the elbow flexors, Child *et al.* [183] used 70 maximal eccentric actions of the knee extensors, while Hellsten *et al.* [185] used 25 min of one-legged eccentric cycling. Moreover, there is no indication in any of these studies that subjects were conditioned to exercise training. It is possible that the very small sample sizes used in these investigations (7–8 subjects) resulted in an inability to detect measurable changes in lipid peroxidation biomarkers. Also, differences in the assay techniques employed across the various studies could be responsible for the mixed results.

Aside from lipid peroxidation, blood protein carbonyls have been reported to be elevated following eccentric resistance exercise [181, 186], with the peak rise in this biomarker occurring from 24- to 48-hours postexercise. Oxidized glutathione has also been reported to increase within the first 2 hours following eccentric exercise [181], as well as at both 4 and 24 hours following eccentric exercise [187]. Lee and Clarkson [188] reported an increase in plasma total glutathione through 120 hours following 50 maximal eccentric muscle actions using the elbow flexors. However, the increase was only observed in subjects with low plasma total glutathione at baseline (<2.5 μM), while subjects with values greater than 3.8 μM demonstrated no change. Finally, DNA oxidation as measured by 8-OHdG was shown to be increased in skeletal muscle following 200 eccentric repetitions using the knee extensors [189]. The increase in 8-OHdG was related to an increase in muscle tissue nitric oxide production, providing evidence that oxidant production in the form of nitric oxide can promote oxidation of DNA. Unlike studies using dynamic resistance exercise, as discussed in Section 4.2.1, in which no changes in 8-OHdG were noted, the study by Radak *et al.* [189] used an extreme volume of exercise (200 eccentric repetitions) and untrained women. It is possible that such an insult, induced in untrained subjects, is necessary to produce measurable DNA oxidation.

4.2.3. *Isometric Exercise*

A few studies have used isometric muscle actions (no movement) as a method to induce oxidative stress, utilizing primarily handgrip exercise. The earliest report comes from Sahlin *et al.* [190] who had subjects perform intermittent knee extension exercise (10 s on, 10 s off) at 30% maximal voluntary contraction for 80 min or until exhaustion was reached. Blood samples and biopsies of the quadriceps muscle were taken before exercise and at 20-min intervals for the entire duration of the exercise session (e.g., 20, 40, 60, 80 min) and analyzed for MDA and glutathione status. No changes were noted for variables in either blood or muscle, with the exception of an increase in total glutathione in blood (greatest at 80 min). However, considering that the exercise intensity was relatively low, it is possible that the stimulus for RONS production was too weak, leading to no measurable change in the biomarkers studied. More recently, Sahlin *et al.* [191] reported that isometric knee extension exercise performed at a higher intensity (five sets to exhaustion using 66% of maximal voluntary force) failed to increase blood TBARS levels, but induced mitochondrial dysfunction in the affected skeletal muscle tissue.

Other studies using isometric handgrip exercise have noted increased lipid peroxidation as measured by lipid hydroperoxides [119], MDA [192], and TBARS [193], while others have noted no change in TBARS [60]. Protein oxidation has only been measured following isometric exercise in one study to date [119] and found to be increased immediately postexercise, returning to basal values by 1-hour postexercise.

Changes in oxidative stress biomarkers following isometric exercise have been transient, and likely due to increased metabolic demand, increased ATP requirements, production of lactate, and acute periods of ischemia followed by reperfusion. No studies have systematically investigated these factors in relation to oxidant production from isometric exercise. While only one of the above investigations extended measurements beyond 60-min postexercise [191], it is doubtful that a longer time course of study would provide differing results, as isometric exercise likely does not induce significant muscle injury resulting in delayed increases in RONS and oxidative stress. The short-lived rise in certain oxidative stress biomarkers following isometric exercise is characterized well in the study by Rodriguez *et al.* [192] in which MDA was elevated immediately and 1-min postisometric handgrip exercise, but returned to basal levels by 3-min postexercise.

4.2.4. *Sprint and Jump Exercise*

Two final forms of anaerobic exercise studied in relation to oxidative stress are sprint and jump exercise. While only one study has used multiple jumps to induce oxidative stress [194], noting no change in MDA in either blood or

muscle, others have used cycle [57, 59, 120, 195, 196], running [100, 197–199], and swim [99] sprints.

Lipid peroxidation has been shown to increase in blood following sprint exercise in some [100, 196, 198] but not all studies [57, 59, 120]. As with other forms of anaerobic exercise, inconsistencies in findings may be related to the overall volume and intensity of exercise, in addition to variance in the assays used. Inal *et al.* [99], while not studying lipid peroxidation, noted a decrease in whole blood glutathione following a 100-m swim sprint, suggesting increased oxidative stress.

Protein oxidation has only been measured in two investigations involving sprinting [59, 120], with an increase noted in one of these investigations [120]. Two studies have measured DNA oxidation following sprinting [120, 197], with only that of Schiffl *et al.* [197] noting an increase. In this study, subjects performed two exhaustive sprints in which blood samples were taken before exercise and for the 2 days postexercise. The number of micronuclei in 3000 binucleated blood lymphocytes was assessed as a marker of DNA damage, and noted to be increased above basal levels at both 24 and 48 hours postexercise.

Aside from human investigations using sprint exercise, a few animal studies have been conducted. Alessio and Goldfarb [101] were the first to report increased TBARS and lipid hydroperoxides in rat skeletal muscle immediately following a 1-min sprint performed at 45 m/min, suggesting that a minimal volume of sprint exercise can increase lipid peroxidation in skeletal muscle. Increased skeletal muscle (but not liver) TBARS was also noted in mice following 15 sprints at 35 m/min for 30 s [200], suggesting a tissue-specific response. Protein oxidation has also been reported in the lungs of rats following sprint exercise [201].

4.2.5. *Sports Play*

Although the majority of studies have used controlled laboratory experiments to investigate exercise-induced oxidative stress (especially with regards to anaerobic exercise), a few studies have focused on sports play, involving predominantly anaerobic work. In relation to acute bouts of play, MDA and protein carbonyls were noted to increase in trained climbers following a climb to exhaustion [98], representing a combination of isometric and dynamic exercise. Increased markers of lipid peroxidation have been noted following single games of American profession football (total lipid peroxides and the titer of autoantibodies against oxidized LDL [31]) and rugby (conjugated dienes and TBARS [179]). In a recent study, Ascensao *et al.* [202] reported an increase in MDA and protein carbonyls in professional motocross athletes following a simulated competitive motocross race. Although not in relation to an acute exercise bout, Schroder *et al.* [58] noted no change

in MDA in professional basketball players following an 8- to 10-day period of training with sessions lasting approximately 90 min. In opposition to these findings, Schippinger *et al.* [31] noted an increase in total peroxides in professional football players during the course of the 5-month competitive season.

4.2.6. *Summary of Anaerobic Exercise*

Based on the available evidence, although results are somewhat mixed, it appears that anaerobic exercise, whether involving mixed concentric–eccentric muscle actions (involving both resistance exercise and sprinting), eccentric only muscle actions, or isometric muscle actions can induce oxidative stress, as measured primarily by lipid, protein, and DNA oxidation. This is the case in several tissues, as the presence of modified macromolecules has been noted in skeletal muscle, blood, and lung tissue. The exercise intensity, the time of postexercise sample collection, the muscles of action (e.g., quadriceps compared to forearms), as well as the site of blood sampling/tissue extraction in relation to the active musculature all may have a role in the explanation of findings. Because such a wide variety of research designs have been used, specific conclusions regarding the exact extent and location (e.g., blood, skeletal muscle) of oxidative macromolecule damage following anaerobic exercise are difficult to compose at the present time. Additional study is needed in this area before answers to these questions can be accurately provided.

4.3. Gender Differences in Exercise-Induced Oxidative Stress

Estrogen appears involved in the upregulation of longevity-associated genes [203] and has antioxidant properties *in vitro* [204]; hence, may provide protection against RONS production. Therefore, it has been suggested that women may be better protected from oxidant attack and experience less exercise-induced oxidative stress compared to men [205, 206]. However, both animal and human studies have failed to support this notion. For example, an acute bout of exercise performed by male and female rats resulted in significant but equal tissue oxidative stress in both genders, as indicated by tissue glutathione status [207]. Ginsburg *et al.* [208] found that women were actually more susceptible to lipid peroxidation than men following completion of an Ironman triathlon, and Kaikkonen *et al.* [209] reported no gender difference in exercise-induced oxidative stress in response to a 42-km run. We have recently reported that protein carbonyls are increased in a similar manner for both men and women following three different duration exercise bouts (30, 60, and 120 min) performed at 70% VO_{2max} [91]. Our most recent work indicates that while women have higher resting blood glutathione and vitamin E levels compared to men, protein,

lipid, and glutathione oxidation increase to a similar extent for both men and women following exercise [210]. Moreover, it has been reported that women with higher estrogen levels (those taking oral contraceptives) demonstrated a delayed strength recovery following the performance of a bout of eccentric muscle actions compared to women not using estrogen [211]. While not measuring oxidative stress markers in this investigation, these data fail to support the hypothesis that estrogen is protective against muscle-related injury, which may be associated with RONS production. Related to this, we have noted typical values for resting and exercise-induced oxidative stress biomarkers in women using oral contraceptives [59, 181], and no differences in resting and exercise-induced oxidative stress biomarkers in women during both the follicular and luteal phases of the menstrual cycle, despite differences in estradiol [212]. Moreover, it has been recently reported that menstrual cycle status has no influence on LDL oxidizability [213]. Therefore, based on the available evidence, it appears that no differences exist between genders or between women either using or not using exogenous estrogen, in regards to exercise-induced oxidative stress. Despite this, due to the possibility that estrogen may provide *in vivo* antioxidant protection, many studies of exercise-induced oxidative stress have chosen to use men exclusively as subjects.

4.4. Exercise-Induced Oxidative Stress in Aging and Diseased Populations

While aging alone is associated with increased oxidative stress [22], acute exercise may lead to age-dependent increases in oxidative stress biomarkers. This has been noted previously by Navarro-Arevalo and Sanchez-del-Pino [214] who reported a greater increase in skeletal muscle TBARS following exhaustive treadmill exercise in older (24–27 months) compared to younger (3–5 months) rats. As expected, basal TBARS levels were also higher in older rats. In support of these findings, Bejma and Ji [135] reported greater RONS production (measured by the oxidation rate of dichlorofluorescin, DCFH) both at rest and following exhaustive treadmill exercise in skeletal muscle of older female rats (24 months) compared to younger rats (8 months). However, these investigators noted a similar increase in MDA and no increase in protein carbonyls in younger and older animals following exercise. In a recent human study, Sacheck *et al.* [108] reported a similar increase in isoprostanes in younger (26.4 ± 3.3 years) and older (71.1 ± 4.0 years) men following a downhill run for 45 min at 75%VO_{2max}. MDA increased in older men immediately postexercise, with no increase noted in younger men. Leukocyte 8-OHdG was unaltered in either group; however, older men had higher basal levels compared to younger men. Taken together, the above data indicate that age may impact the oxidative stress response to

acute exercise. As with diseased conditions, it appears that elevations in basal levels of oxidative stress biomarkers are often associated with an exacerbation in exercise-induced oxidative stress biomarkers; however, this may be biomarker specific, highlighting the need for inclusion of multiple biomarkers within the study design.

Although some studies have focused on age-related differences in exercise-induced oxidative stress, it should be noted that most studies have focused on younger, healthy subjects. In particular, those who can withstand the extreme intensity and duration of many of the exercise protocols, as well as the potential for extreme discomfort and muscle injury that is often associated with such protocols. However, due to the likely role of RONS in a variety of disease conditions, as well as the prevalence of regular exercise as a method of treating many diseases, investigators have begun to study exercise-induced alterations (both acute and chronic) in oxidative stress with respect to disease, in particular conditions related to cardiovascular disease (e.g., obesity, diabetes). The majority of these studies have used aerobic exercise.

Exercise-induced oxidative stress has been the focus of four investigations in which obese patients have been used as subjects [215–218]. In the first investigation of its kind, obese subjects were reported to experience an exacerbation in postexercise lipid peroxidation, following both acute resistance and aerobic exercise [215]. These findings were supported in a similar investigation using obese postmenopausal women [216]. In follow-up studies it was shown that obese patients experience an exaggerated increase in lipid peroxidation compared to nonobese controls in response to exercise, and that the increase can be attenuated with the use of antioxidant (800 IU vitamin E, 500 mg vitamin C, 10 mg beta-carotene) supplementation [217] and 6 months of resistance training [218]. These last two studies provide evidence that both antioxidant supplementation and regular resistance training exercise can attenuate the rise in oxidative stress following acute bouts of physical work.

Patients with cardiovascular disease [219–221] and diabetes [222–224] have also been the focus of study in this area. It was reported that MDA levels were higher at rest and following a symptom-limited exercise test in patients with hypercholesterolemia [219] and heart failure [221], while no change in MDA was noted following similar testing in heart transplant recipients [220]. It is possible that the training status of the heart transplant recipients (endurance training 3 hours/week) allowed for an upregulation in antioxidant defense, minimizing lipid peroxidation to exercise. This is underscored by findings indicating that exercise training can decrease susceptibility to oxidative stress in patients with coronary artery disease [225] and increase gene expression for key antioxidant enzymes in patients with chronic heart failure [226].

Although not considered a disease, but rather an activity that may promote disease, cigarette smoking exacerbates RONS formation and presents a significant oxidant stress *in vivo* [227]. It is well documented that cigarette smokers have elevated biomarkers of oxidative stress compared to nonsmokers at rest [228], which may represent a potential mechanistic link between cigarette smoking and cardiovascular disease [229]. The increased oxidative stress observed in smokers is due in part to both increased oxidant production by the cigarette smoke [230], as well as the lower blood antioxidant capacity routinely observed in smokers [227]. It is plausible that the addition of other RONS generators can further promote oxidative stress in cigarette smokers. Related to the discussion of exercise and oxidative stress, we have recently reported an exacerbation in exercise-induced oxidative stress in cigarette smokers compared to nonsmokers [131].

Aside from the above conditions, diabetes has been the focus of other work. Two studies have investigated exercise-induced oxidative stress in type 1 diabetics, with similar increases observed for TBARS and GSSG compared to healthy control subjects [223], as well as for lipid peroxidation and radical generation following exercise [222]. In contrast, sedentary type 2 diabetics demonstrated an increase in TBARS following a graded exercise test as compared to active type 2 diabetics and healthy controls [224], suggesting again that regular physical activity can attenuate exercise-induced oxidative stress, even in those subjects with known disease. Findings such as these may be the basis for future intervention studies using exercise as a method of increasing endogenous antioxidant protection and decreasing oxidant production, in an attempt to possibly influence disease onset and progression.

4.5. Summary of Exercise and Oxidative Stress

From the early work of Dillard *et al.* in 1978, several investigators have reported increased oxidative stress following acute bouts of strenuous physical exercise. While not apparent in all studies, these findings have been reported for both aerobic and anaerobic exercise. The degree of change in oxidative stress and antioxidant status biomarkers is dependent on a number of factors related to the actual exercise itself, the subject population, as well as the analytical procedures used in testing the outcome measures. It should be understood that many analytical procedures, although routinely employed, have not been extensively validated. As such, different laboratories may use widely diverse procedures to measure the same biomarker, often resulting in a high degree of variance from one laboratory to another. Indeed, this lack of validation could be responsible for differences in findings across studies.

Because cellular status is constantly changing and involves an array of regulatory systems, inclusive of changes in both the generation and handling of RONS, caution should be used when drawing conclusions from studies in which elevations in isolated oxidative stress biomarkers, in isolated tissues, have been reported. Moreover, although RONS are often viewed in a negative manner, production that does not exceed some unknown threshold appears necessary for several important biological processes, as discussed in Section 3.1. Perhaps most applicable to the theme of this chapter is the upregulation of endogenous antioxidant defense as a result of acute RONS production. Increased antioxidant defense may be one additional mechanism allowing for improvements in health, possibly having implications related to minimizing both the initiation and progression of certain diseases. This is certainly an exciting area for future research—the use of regular exercise as a method of enhancing health and possibly minimizing disease via upregulation of antioxidant defense. While it is possible that elevations in RONS in response to acute exercise may suggest a detrimental response within cells, the long-term biological significance of such acute changes has yet to be determined. Only more extensive, longitudinal research studies will provide information related to whether these acute changes in oxidative status should be viewed as positive, or whether such changes will manifest in biological maladaptations leading to ill-health and impaired physical functioning.

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HUMAN TOTAL SERUM N-GLYCOME

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1. Abstract

The human total serum N-glycome (TSNG) is the representation of the serum glycoproteins *N*-glycosylation. The study of *N*-glycosylation in clinical biochemistry laboratories is emerging as a new assay for diagnosis of congenital and acquired diseases. This review describes the *N*-glycosylation of serum glycoproteins and its contribution to the serum N-glycome, the different

0065-2423/08 $35.00
DOI: 10.1016/S0065-2423(08)00402-2

protocols allowing the achievement of an *N*-glycosylation profile, the variations of this profile in diseases, and finally the perspective of applications of the TSNG in the exploration of congenital disorders of glycosylation (CDG), hepatic cirrhosis, inflammatory disorders, autoimmune diseases, and cancers.

2. Introduction

The glycome, by analogy with genome and proteome, is the representation of the total glycosylation of a human being [1]. Glycosylation of proteins is highly heterogeneous process with more than 37 sugar–amino acid combinations existing [2]. The human TSNG describes only one of these sugar–protein associations, that is, the β-glycosylamine linkage of *N*-acetyl glucosylamine to the amino acid residue, asparagine. Despite being the most common posttranslational modification of plasma proteins, it represents only a minor component of the human glycome [2].

Following the first description of a CDG by Jaeken and Matthijs in 1980 [3], more than 20 genetic disorders of *N*-glycosylation have now been identified. Although all organs are affected, there is a predilection for central nervous system involvement. According to the severity of these diseases and the studies of knockout mice, the importance of *N*-glycosylation has been demonstrated. The time has passed when the roles of N-glycans were confined to protease resistance, protein solubility, and when sugars were considered as a simple "decoration," and today their roles span a variety of functions from cell recognition and adhesion, cell migration, host defense to molecular trafficking and clearance [4].

Nevertheless, in clinical biochemistry laboratories, the study of *N*-glycosylation has still not emerged as a diagnostic or prognosis tool. During the last decade, novel analytical procedures allowing a rapid, reproducible and high-throughput analysis of the serum N-glycome have been developed and their application in diagnosis is only beginning to be described.

In this chapter, we will focus on the *N*-glycosylation of serum glycoproteins that contributes to the serum N-glycome, on the different protocols allowing the achievement of an *N*-glycosylation profile, on the variations of this profile in diseases, and finally on the perspective of applications of the TSNG in clinical biology.

3. *N*-Glycosylation of Serum Glycoproteins

3.1. Structure of N-Glycans

All N-glycans are characterized by the presence of a branched pentasaccharidic core structure consisting in three residues of mannose (Man) and two residues of *N*-acetylglucosamine (GlcNAc) (Fig. 1). The core is linked to

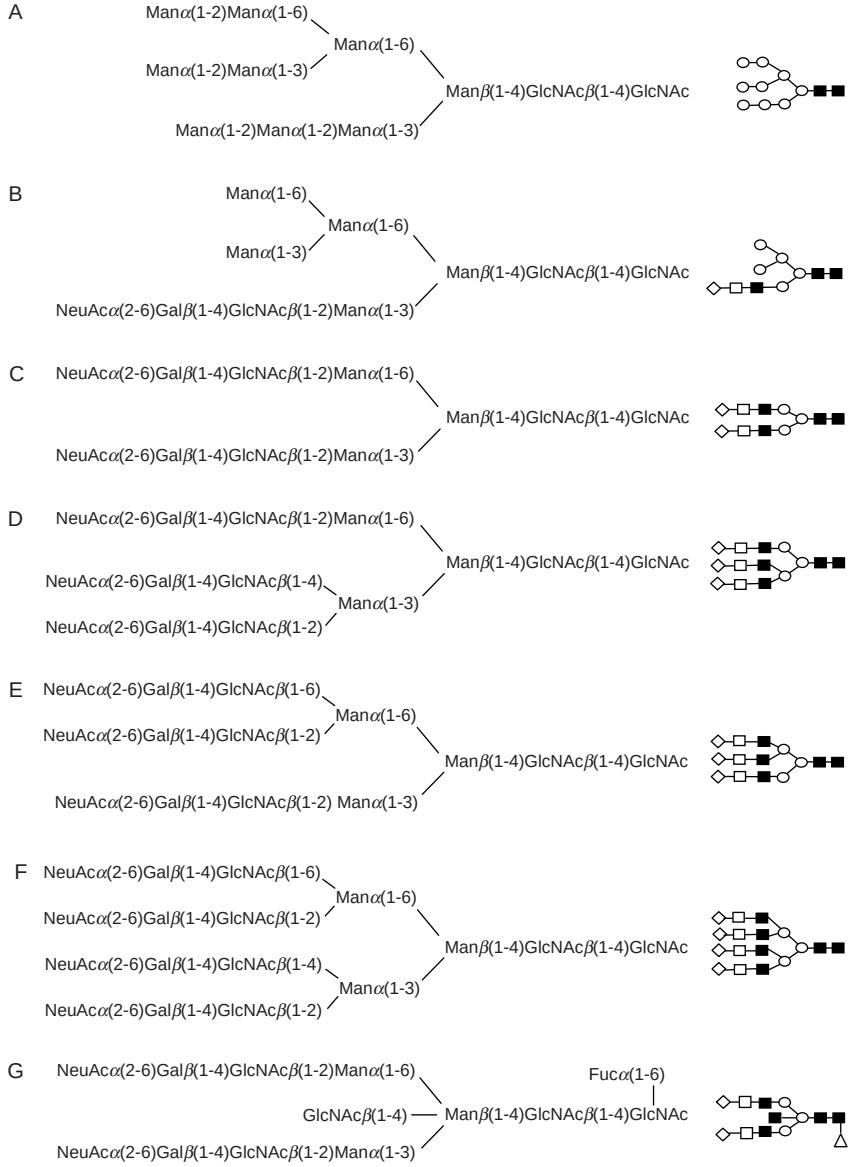

FIG. 1. Structures of the main types of human N-glycans and characteristic "complex" oligosaccharides: (A) oligomannosidic-type structure; (B) hybrid-type structure; (C–G) "complex-type structures; (C) biantennary disialylated; (D–E) triantennary trisialylated; (F) tetraantennary tetrasialylated; (G) bisected biantennary disialylated core α1,6-fucosylated oligosaccharide. Symbols for the structural formulas are defined as follow: solid square, GlcNAc; open square, Gal (galactose); open circle, Man (mannose); triangle, fuc (fucose); open diamond, NeuAc (*N*-acetylneuraminic acid).

the peptide backbone by a GlcNAc-βAsn bond, the asparagine being part of a consensus sequence Asn-X-Ser/Thr. From this pentasaccharidic core, extensions are made on the mannose residues at the nonreducing end. These extensions define three types of N-glycans: (1) high mannose structures when mannose residues are only added (Fig. 1A), (2) hybrid structures when mannose residues are added on the Man α1,6 arm and at least one GlcNAc residue on the Man α1,3 arm (Fig. 1B), and (3) "complex" structures when GlcNAc residues are added on both Man α1,3 and Man α1,6 arms (Fig. 1C–G).

The extension of the core defines the backbone of the oligosaccharide, represents the first level of diversity of the "complex" N-glycans, and consists of a number of antennae (i.e., bi-, tri-, tetra-, pentaantennae; Fig. 1C–E) and a number of "*N*-acetyllactosamine" units repeat.

The periphery of an N-glycan consists in the adjunction of fucose (Fuc), sialic acid, galactose (Gal), *N*-acetylgalactosamine (GalNAc), phosphate and sulfate residues. The addition of these residues results in antigenic determinants such as the blood group antigens or other tissue-specific antigens. The linkage of these different components is also variable; for example, sialic acid can be linked α2,3 or α2,6 to a galactose residue of the backbone. In fact, to be precise, even sialic acid does not relate to one monosaccharide but to a family of more than 40 members [5].

This results in an infinite possibility of structures. It would be tempting to consider this phenomenon as structural "noise" or "decoration," but the finely tuned biosynthesis and the particular biological roles of N-glycans demonstrate the contrary [4].

3.2. Biosynthesis

The biosynthesis of N-glycans is a complex cotranslational process. The first step is the preassembly of a tetradecasaccharide on a lipid intermediate, the dolichol phosphate (Dol-P). This intermediate is first assembled on the cytosolic face of the endoplasmic reticulum (ER) (steps 1–3 in Fig. 2), and, after translocation (step 4, Fig. 2) to the luminal face, the assembly of the complete lipid oligosaccharide can be achieved (step 5–8, Fig. 2). This tetradecasaccharide is transferred by the oligosaccharidyl transferase to the nascent glycoprotein on the asparagine residue of a consensus sequence Asn-X-Ser/Thr (step 9 in Fig. 2). In the ER, this oligosaccharide is trimmed by a complex process, involving deglucosylation, reglucosylation, and demannosylation (steps 10–12). This process is involved in the ER quality control and, only when the glycoprotein is properly folded, the N-glycan biosynthesis can continue in the Golgi complex and the *trans*-Golgi network [6–8].

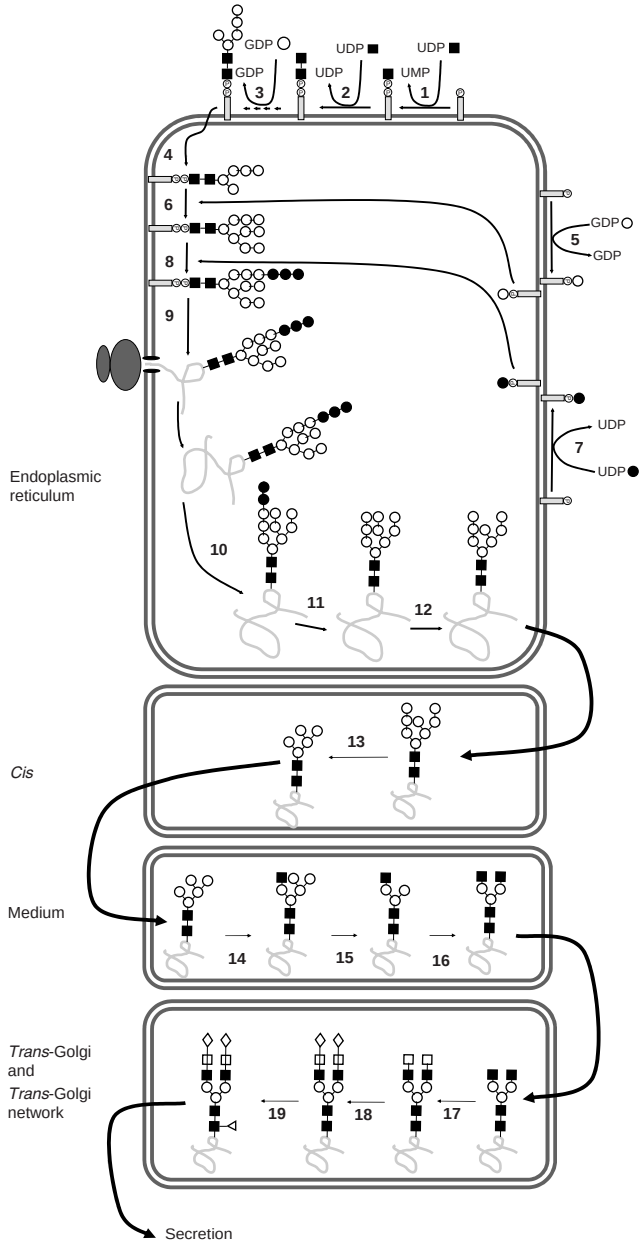

FIG. 2. Biosynthesis of serum glycoprotein N-glycans. The reactions are catalyzed by the following enzymes: (1) UDP-GlcNAc: Dol-P GlcNAc-P transferase; (2) UDP-GlcNAc: Dol-P-GlcNAc-P GlcNAc transferase; (3) mannosyl transferases GDP-Man dependent; (4) flippase;

In the *cis*- and medium-Golgi, the high mannose N-glycan is further trimmed and the action of mannosidases and *N*-acetylglucosaminyl transferases (GlcNAc-T) directs the oligosaccharide in various pathways (high mannose, hybrid, or complex). Glycan diversity is mostly generated at this stage, as six different GlcNAc-Ts can act on the mannose residue of the core, creating the branching heterogeneity. All the transferases act sequentially, in a specific manner, with acceptor specificity and with the classical rule ''one enzyme, one glycosidic linkage.'' They also have a tissue-specific expression; the protein backbone influences the glycosylation (i.e., steric hindrance; mannose 6-phosphorylation for the addressing of lysosomal enzymes [6]). Galactosyl-, fucosyl-, and sialyltransferases complete the N-glycan structure that finally results in a mature membrane bound or a secreted glycoprotein (steps 13–19, Fig. 2) [6].

Glycan sequence is not directly dependent of a DNA template. The structure specificity relies on the expression of various proteins such as enzymes (i.e., glycosyltransferases, enzymes of the monosaccharide metabolism, glycosidases), transporters (i.e., nucleotide sugar transporters), and flippase. Defects in glycosylation, encountered in CDG or in knockout mice, have permitted the identification of other proteins involved in glycosylation such as the conserved oligomeric Golgi 7 (COG7) protein, which affects the localization and traffic of Golgi proteins [9].

3.3. Serum Protein *N*-Glycosylation

The TSNG is the sum of hundreds individual glycoprotein N-glycomes, secreted by or leaking from all the different tissues of the body. The number of high-abundance glycoproteins is relatively small, and immunoglobulins (IgG, IgA, and IgM), transferrin, and α2-macroglobulin represent together approximately 75% of the total serum glycoproteins. As the liver and the immunoglobulin-secreting plasma cells are the main source of the serum glycoproteins, any alterations in the liver and/or B-lymphocyte physiology during diseases might influence the *N*-glycosylation mechanisms leading to a different distribution of the glycoforms with modified biological properties.

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(5) dolichol-P-Man synthase; (6) mannosyl transferases Dol-P-Man dependent; (7) dolichol-P-Glc synthase; (8) glucosyltransferase Dol-P-Glc dependent; (9) oligosaccharyl transferase; (10) α1,2-glucosidase I; (11) α1,3-glucosidase II; (12) endoplasmic reticulum α1,2-mannosidase; (13) Golgi α-mannosidase I; (14) GlcNAc transferase I; (15) Golgi α-mannosidase II; (16) GlcNAc transferase II; (17) Gal transferase; (18) sialyltransferase; (19) fucosyltransferase. Symbols for the structural formulas are defined as follow: solid square, GlcNAc; open square, Gal (galactose); open circle, Man (mannose); triangle, fuc (fucose); open diamond, NeuAc (*N*-acetylneuraminic acid); dolichol, gray rectangle; phosphate, circled P.

The roles of the glycan moiety of serum glycoproteins are extremely diverse; these can be classified in two groups: (1) alterations of the general properties of the glycoprotein such as the modification of the physicochemical properties (solubility, stability, conformation), the half-life of the glycoconjugate, and the resistance to proteases; and (2) control and regulation of biological processes by protein–saccharide interactions [4].

3.3.1. *Immunoglobulin G* N-*Glycosylation*

Immunoglobulin G (IgG) are the most abundant glycoproteins in the serum (800–1600 mg/100 ml), synthesized by activated B lymphocytes and comprises two heavy and two light chains which are composed of variable and constant domains. Limited proteolytic cleavage of IgG results in two fragments, the Fab (fragment antigen binding) and the Fc (fragment crystallizable). The carbohydrate moieties account for 2–3% of the molecular weight and correspond mainly to N-glycans located on Asn-297 of the C_H2 domain of each heavy chain subunit. The variable region of IgG may be also glycosylated. The largest oligosaccharide possesses 13 sugar residues and corresponds to a biantennary disialylated "complex-type" N-glycan with a bisecting β1,4-GlcNAc residue and a fucose residue α1,6-linked to the core GlcNAc (Fig. 1G) [10–14]. More than 30 different structures have been identified. These correspond to the tridecasaccharide lacking one or more sugar residues and are distributed into four groups: the sialylated oligosaccharides (14%) and three types of neutral oligosaccharides, namely, the agalactosylated structures terminated by a GlcNAc residue on both antennae (G_0-IgG, 35%), the monogalactosylated (G_1-IgG, 35%), and the structures in which both antennae are terminated by a galactose residue (G_2-IgG, 16%) [13, 14].

The two N-glycans of the Fc fragment modulate the effector functions of IgG, especially the activation of the complement system and the binding to Fc gamma receptor (FcγR) present on phagocytes.

In contrast to galactosylated IgG (G_1-IgG and G_2-IgG), agalactosylated IgG are able to activate the complement system via the lectin pathway. The terminal GlcNAc residues of the G_0-IgG are recognized by the mannan binding lectin (MBL), a member of the collectin family that has an important role in innate immunity: the activation of this pathway can trigger rapid-enhanced phagocytosis, and is also involved in the physiopathology of rheumatoid arthritis [15, 16].

Glycosylation is required for optimal binding to FcγR [17] and the chemical composition of the glycan modulates the affinity. The fucose residue α1,6-linked to the core GlcNAc present on 70% of the IgG N-glycans decreases the affinity of the antibody to FcγRIIIa and results in a 100-fold diminished antibody-dependent cellular cytotoxicity (ADCC) [18].

Recent studies have shown the importance of Fc sialic acids in the interaction with FcγR. Terminal sialic acids lower the IgG affinity toward their lymphocytic receptors, and as a consequence upon antigenic contact, the loss of sialic acid switches the immunoglobulin properties from anti-inflammatory to pro-inflammatory [19, 20].

The first disease related to the modification of IgG glycosylation was rheumatoid arthritis, described in 1985 [11]. The distribution of the IgG glycoforms in the serum of these patients showed an increase in the percentage of the agalactosylated G_0-IgG molecules. Subsequently, a relative increase of agalactosylated IgG has been observed in numerous inflammatory and autoimmune diseases, including tuberculosis [21], Crohn's disease [22], celiac diseases [23], psoriatic arthritis, periodontal disease [24], infection with HIV [25], systemic vasculitis associated with antineutrophil cytoplasmic antibodies [26], and cancer [27]. The agalactosylation of IgG appears as a pro-inflammatory mechanism common to numerous inflammatory and autoimmune diseases. A decreased IgG sialylation has been observed following immunization with a test antigen; this could correspond to a switch from a steady anti-inflammatory state to a protective inflammatory state upon immunization [19]. The impact of the glycan structures found on the Fc of the IgG appears to modulate the effector properties of the molecule.

3.3.2. *Immunoglobulin A* N-*Glycosylation*

In human serum there is 200–400 mg/100 ml of immunoglobulin A (IgA) existing as two isotypes: 90% of IgA1 and 10% of IgA2. All IgAs contain two N-glycans at position 263 of the C_H2 and 459 of the C_H3 domain. IgA2 contains two or three additional N-glycans. Thirty percent of the Fab fragment of IgA1 is N-glycosylated. IgA1 contains O-linked oligosaccharides, glycans linked to the peptide backbone through a hemiacetalic linkage of a GalNAc to the hydroxyl group of a serine or a threonine residue in the hinge region. The structures of the N-linked oligosaccharides of IgA1 are distributed into biantennary (86%) and triantennary structures (14%). More than 90% of the glycan is sialylated and less than 2% is agalactosylated. A bisecting GlcNAc is present in 24–40% of the N-glycans, indicating possible individual variations [28–30]. α1,6 core fucose is found in 32% of the N-linked oligosaccharides [14]. As compared to IgG N-glycans, in IgA1 the glycans are more sialylated, more branched, and less fucosylated with a reduced number of bisecting GlcNAc. As for IgG, the glycan modulates the Fc fragment binding to MBL [31] and to the asialoglycoprotein receptor (ASGP-R), which clears from the serum the desialylated galactose-terminated glycoproteins. ASGP-R binds to galactose and GalNAc-terminated oligosaccharides and removes IgA2 not the sialylated structures of IgA1 from the serum, explaining their predominance in the serum [32].

Abnormal *O*-glycosylation of IgA has been described in IgA nephropathy; the IgA1 O-glycans being less galactosylated and sialylated. This disease is characterized by a deposit mainly composed of polymeric IgA1 in the glomerular mesangium. The *N*-glycosylation of these polymeric IgA1 is also modified with the presence of oligomannosidic N-glycans, which could participate in the inflammation by activation of the lectin pathway of the complement [33].

Sjögren's syndrome is an autoimmune disease associated with an increased level of serum IgA. The IgA1 N-glycans present in this disease, have an increased sialylation which reduces their clearance by the ASGP-R [34].

3.3.3. *Immunoglobulin M* N-*Glycosylation*

Serum immunoglobulin M (IgM), a polymeric immunoglobulin, is a pentameric molecule composed of 10 heavy chains, 10 light chains, and 1 J chain, present in the serum at a concentration of 50–200 mg/100 ml. It is heavily glycosylated with five *N*-glycosylation sites present on the heavy chain and on the J chain. Complex-type oligosaccharides are found on the first three glycosylation sites and on the J chain. Oligomannosidic oligosaccharides are found on the fourth and fifth glycosylation sites at the C-terminus. Eighty-one percent of the N-glycans is sialylated, 85% core fucosylated, 52% present a bisecting GlcNAc residue, and only 4% present a non-galactosylated GlcNAc residue [14, 35, 36]. A molecular model of the pentameric IgM has been proposed in which IgM has two distinct faces, only one of which can bind antigen. The complex-type glycans are located on the antigen-binding site. Upon engagement of the Fab fragment with the antigen, the complex glycan chains are not accessible anymore to the lectin-binding site, and as a consequence the activation of the complement by the lectin pathway by antigen bound IgM pentamers is unlikely [14, 37].

3.3.4. *Transferrin*

Human serum transferrin is a glycoprotein synthesized by the liver and is involved in iron transport between sites of absorption and sites of delivery. It is present in the serum at a concentration of 200–400 mg/100 ml. Transferrin contains two *N*-glycosylation sites at the C-terminal lobe. The two major glycans are the biantennary and triantennary fully sialylated oligosaccharides in a ratio of 85:15. The heterogeneity results from the loss of terminal sialic acid residues and of the presence of core fucosylation [38–40]. The different combinations of the two glycans result in tetra-, penta-, and hexasialylated variants of serum transferrin. The different glycoforms have a similar binding capacity for iron and affinity to the transferrin receptor; only the aglycotransferrin has a diminished iron-binding capacity [41]. Separation and quantification of transferrin glycoforms is currently used in

clinical chemistry laboratories for the diagnosis of chronic alcohol abuse and for the screening of CDG. The presence of a hyposialylated form of transferrin in the plasma of chronic alcohol consumers was described 30 years ago by Stibler and termed carbohydrate deficient transferrin (CDT) [42]. The CDT corresponds principally to the loss of an entire N-glycan chain and a minor fraction to the loss of terminal sialic acids [40]. Abnormal biosynthesis of transferrin N-glycans is seen in CDG, and transferrin isoelectrofocusing is the screening tool for these diseases [43]. In hepatocarcinoma, numerous modifications of transferrin glycosylation are observed, in particular, the increased branching of the N-glycans and the presence of a bisecting GlcNAc residue [44]. During pregnancy, increased branching and sialylation of transferrin glycoforms are observed [45].

3.3.5. *α2-Macroglobulin*

α2-Macroglobulin is a tetramer with a molecular weight of 720 kDa, found in the serum at a concentration of 200–400 mg/100 ml. Each of the four 180-kDa subunits contains eight *N*-glycosylation sites [46]. The N-glycans are principally of the complex type; approximately 7% oligomannosidic structures are present, especially located on Asn-846. The biantennary structures are predominant (85%) and are fully sialylated or galactose terminated. Triantennary represent 5% of the total N-glycans and approximately 20% of the structures are fucosylated, no bisecting GlcNAc residues are seen in normal pooled serum [47].

α2-Macroglobulin is a protease inhibitor that contains a "bait" region that is cleaved after contact with the proteinase. This cleavage triggers a conformational change that traps the protease, and the resulting complex is cleared from the serum. Recently, α2-macroglobulin has been shown to interact with MBL; this interaction takes place after recognition of foreign proteases at the surface of microorganisms, and triggers enzyme cascades such as the complement [47]. Glycosylation modifications of α2-macroglobulin have been detected in various autoimmune diseases; for example, increased carbohydrate content was found in systemic lupus erythematosus [48], a modified glycosylation has also been suggested to take place in multiple sclerosis [49].

3.3.6. *α1-Antitrypsin*

α1-Antitrypsin is a member of the serine protease inhibitor (serpin) superfamily. It is a 52-kDa glycoprotein containing three N-glycans and is found in the serum at a concentration of 90–200 mg/100 ml. The N-glycans have recently been described, and are of the complex type with 78% of biantennary, 20% of triantennary, and approximately 2% tetraantennary structures. The structures are fully sialylated with less than 1% of galactose-terminated glycans. Fucose is found α1,6-linked to biantennary structures, corresponding to 4% of

the total glycan, whereas in triantennary and tetranatennary glycans fucose is found α1,3-linked to an *N*-acetyllactosamine GlcNAc residue part of sialyl-Lewis^x determinant principally [50]. Several isomers have been observed by isoelectrofocalization [51] due to the combination of different N-linked glycan structures. The N-linked glycan at Asn-83 is alternatively bi-, tri-, or tetra-antennary, whereas glycans at Asn-46 and Asn-247 are principally biantennary. Modification of the distribution of the isoforms is observed in CDG, chronic alcoholism, and hereditary fructose intolerance with α1-antitrypsin underglycosylated with only one or two N-glycans [52–54].

3.3.7. *α1-Acid Glycoprotein*

α1-Acid glycoprotein (AGP), also called orosomucoid, is an acute phase protein, which is synthesized mainly by hepatocytes and belongs to the subfamily of lipocalins, called the immunocalins. AGP is a glycoprotein with a molecular weight of 41–43 kDa, heavily glycosylated (45% carbohydrate by weight) on five *N*-glycosylation sites (Asn-15, -38, -54, -75, and -85) and is found in the serum at a concentration of 50–100 mg/100 ml. The N-glycans are bi- (14%), tri- (38%), or tetraantennary (48%) complex-type oligosaccharides [55]. The antennae are highly sialylated resulting in molecules with very acidic isoelectric points (p*I* 2.8–3.8); the sialic acid is found α2,3- and α2,6-linked to a galactose residue, the α2,3-linked preferentially expressed on tri- and tetraantennae structures. Fucose is found α1,3-linked to an antennae GlcNAc residue that can be part of a sialyl-Lewis^x determinant (NeuAcα2,3 Galβ1,4(Fucα1,3)GlcNAc-) [56]. Thousands of glycoforms can be generated; the five glycosylation sites can be occupied with a bi-, tri-, or tetraantennary structure but there is a specific distribution with triantennary structures principally on Asn-15, biantennary structures on Asn-38, and triantennary on Asn-54. Tetraantennary are principally located on Asn-75 and Asn-85 [55, 57]. The degree of sialylation and/or the number of antennae of the N-glycans affect the immunomodulatory properties of AGP such as lymphocyte proliferation, the induction of IL-1 inhibiting activity in macrophage, and also the inhibition of platelet aggregation [58]. During the acute phase of inflammation, a cytokine-induced decrease in the degree of branching of AGP occurs with an increase of glycoforms with biantennary glycans [57, 59]. Concomitantly, there is a large increase in sialyl-Lewis^x-substituted AGP molecules, a ligand for E-selectin. These soluble molecules, acting as counterreceptors, might influence the selectin-mediated influx of leukocytes into inflamed tissues [59]. In chronic inflammation, there is an increased expression of sialyl-Lewis^x on AGP and of tri- and tetraantennary glycans. On the contrary, during acute inflammation, there is a decrease of tri- and tetraantennary glycans with an increased degree

of fucosylation [57, 60]. Estrogen induces a decrease in fucosylation and an increased branching of the AGP oligosaccharides, a reaction opposite to those found during inflammatory reactions [61].

3.3.8. *Haptoglobin*

Haptoglobin is a multimeric glycoprotein found in the serum at a concentration of 30–190 mg/100 ml, formed by the association of α and β chains, and characterized by a genetic polymorphism. Four *N*-glycosylation sites are present on the β chain. The N-glycans are bi- or triantennary oligosaccharides with terminal sialic acid or galactose residues and the fucose α1,6-linked to the core GlcNAc [62, 63]. Twenty-seven different oligosaccharides have been detected, but not characterized, by high-performance liquid chromatography (HPLC) and their modifications of distribution have been described during Crohn's disease, rheumatoid arthritis, and stomach cancer [64]. In a recent study, the increase of haptoglobin fucosylation during various cancers has also been demonstrated and described as a possible marker of pancreatic cancer [65].

3.3.9. *Other Serum Glycoproteins*

The previously described glycoproteins represent approximately 80–90% of the physiological serum N-glycome; the remainder is represented principally by ceruloplasmin, the complement system, and the apolipoproteins.

Ceruloplasmin contains four *N*-glycosylation sites and is found in the serum at a concentration of 20–60 mg/100 ml. The N-glycan of ceruloplasmin have been characterized as bi- and triantennary complex-type oligosaccharides, sialylated, and α1,3- or α1,6-fucosylated [66, 67].

The complement system is a complex protease cascade with three pathways: the classical, the alternative, and the lectin pathway. The glycosylation pattern of the different proteins depends on the organ of synthesis. The complement components predominantly synthesized in the liver (C1r, C1s, C2, C3, C4, C5, C6, C8, C9, FB, FH, FI, C4 bp) contain complex biantennary oligosaccharides, variably sialylated, and with a low level of fucosylation. C3 contains only oligomannose N-glycans. The components synthesized in the lymphoid organs (C1q, properdin, and C7) are also complex biantennary glycans but the core is predominantly fucosylated [68].

Apolipoprotein B-100 is synthesized by the liver and has a molecular weight of 513 kDa and possesses 16 *N*-glycosylation sites. It contains 6.3% carbohydrate by mass; the N-glycans are either of the complex-type (72%), the oligomannosic-type (23%), or hybrid structures (5%). The complex structures are principally biantennary, neutral, mono- or disialylated with the presence of traces of triantennary oligosaccharides. No fucosylated structure has been found. The distribution of the N-glycans has been shown to

be highly conserved in normal subjects and in selected individuals known to be at high risk to atherosclerosis development [69, 70]. Apolipoprotein(a) contains N- and O-linked glycans; the two principal N-glycans are complex mono- or disialylated biantennary oligosaccharides [71].

4. Determination of the TSNG

In the past decade, the emergence of novel technologies has permitted the development of new methods for the determination of the TSNG. For these methods, to be used in clinical chemistry laboratories, they need to fulfill some specific requirements such as being high throughput, sensitive, reproducible, and easy to implement. Callewaert *et al.* were the first to develop such a protocol and applied it to the exploration of CDG and liver cirrhosis diagnosis [72–75]. The protocol is always based on the same three steps: cleavage of serum glycoprotein N-glycans, purification of the released oligosaccharides, and analysis of the N-glycome (Fig. 3).

4.1. Cleavage of the N-Glycans

N-linked oligosaccharides can be released from the serum glycoproteins by two types of methods: chemical or enzymatic.

Hydrazinolysis was one of the first methods applied to the quantitative liberation of intact N-linked oligosaccharides in an unreduced form and it has been automated [76]. This method has been extensively utilized for structural analysis, but the parameters of the reaction conditions need to be defined precisely. Furthermore, moisture in the samples and in the anhydrous hydrazine has to be avoided to prevent degradation of the N-glycan. For practical reasons, enzymatic deglycosylation is preferred.

Peptide-*N*-(*N*-acetyl β-glucosaminyl)asparagine amidase or PNGase F (EC 3.5.1.52) hydrolyzes the glycosylamine linkage releasing the unreduced oligosaccharide while the asparagine is converted to aspartic acid. The enzyme, which cleaves all N-linked oligosaccharides present in humans, has a broad specificity. It is active between pH 7.5 and 9.5 with an optimum pH of 8.6 [77].

The seric glycoproteins (serum 10 μl) are first denatured to break the polypeptide backbone tridimensional structure and to render all the glycans accessible. Usually the following conditions are used: a thermal denaturation (100 °C, 10 min) in the presence of sodium dodecyl sulfate (SDS) (0.5%) and 2-mercaptoethanol (1%). Due to SDS inhibition of PNGase activity, a non-ionic detergent is added (Nonidet P-40) to counteract the SDS effect. Usually 500 units of PNGase F are added and the digestion is performed at 37 °C for 3 hours. Accessibility to all serum glycoprotein

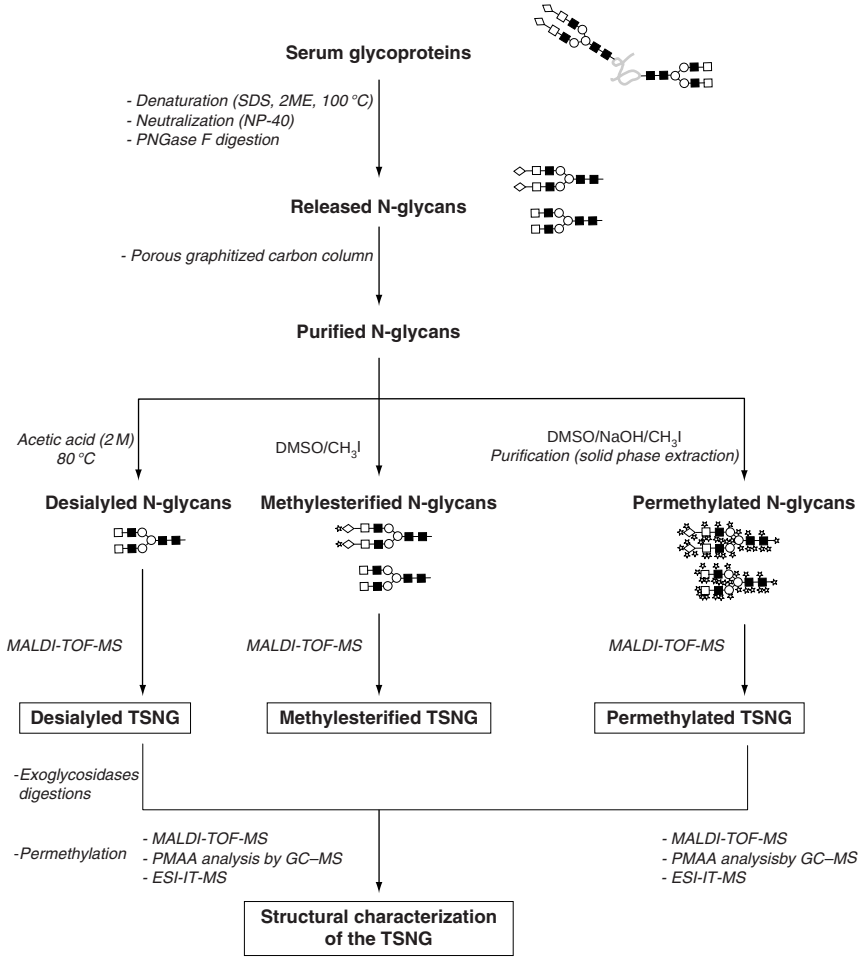


FIG. 3. Strategy of TSNG determination and structural characterization. Symbols for the structural formulas are defined as follow: solid square, GlcNAc; open square, Gal (galactose); open circle, Man (mannose); open diamond, NeuAc (*N*-acetylneuraminic acid); star, methyl group.

N-glycans can be achieved also by protease degradation (trypsin, chymotrypsin, pronase) [78], or by reduction/alkylation of the sample [79, 80]. An alternative approach has been used by Papac *et al.* in which the sample is first immobilized on a polyvinylidene difluoride (PVDF) membrane and then denatured by reduction/alkylation [81].

4.2. Purification of the Released Oligosaccharides

The released oligosaccharides are purified by solid phase extraction, using either hydrophilic chromatography (i.e., Sepharose 4B) [82], hydrophobic chromatography (C18) [80], or adsorption on porous graphitized carbon (PGC) [83, 84]. PGC has the advantage of purifying neutral and sialylated oligosaccharides in a single step; the column is washed with an acetonitrile/water (1/3 v/v) containing 0.1% trifluoroacetic acid (TFA), equilibrated in water. The enzymatic digestion diluted in water (1/20 v/v) is then loaded on the column, and washed with water and the fraction containing the oligosaccharides is eluted with acetonitrile/water (1/3 v/v) containing TFA 0.1% [84].

4.3. Analysis of the N-Glycome

The analysis can be performed with or without derivatization on native or desialylated oligosaccharides.

4.3.1. *Analysis of Oligosaccharides without Derivatization*

Direct analysis of the purified free oligosaccharides can be performed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The sialylated oligosaccharides give multiple ions due to salt adducts [82, 85] and there is an extensive fragmentation of sialic acid residues that necessitates the use of linear mode MALDI-TOF to avoid separating the metastable products. For these reasons, it is preferable to study sialylated oligosaccharides after derivatization or desialylation.

4.3.2. *Analysis of Desialylated Oligosaccharides without Derivatization*

Desialylation of the TSNG reduces the diversity of the oligosaccharides and facilitates the analysis of quantitative variations. Release of sialic acids can be performed either by acid hydrolysis with acetic acid (2 M) at 80 °C for 2 hours or enzymatically by the sialidase of *Arthrobacter ureafaciens* [86]. The neutral N-glycans (100 pmol) are cocrystallized with the 2,5-dihydroxybenzoic acid (DHB) (10 mg/ml in acetonitrile/water/TFA; 300/699/1 v/v/v) as matrix, the mass spectrum being recorded in reflectron mode [83]. The spectra obtained give the distribution of oligosaccharides and their chemical composition. The signal strength of the glycan ionized by MALDI reflects accurately the amount of each component of the TSNG [87], and relative quantification is easily made by reference to a 100% signal corresponding to the main component. Usually, it corresponds to the $[M+Na]^+$ ions at m/z 1664 corresponding to a desialylated biantennary glycan. Furthermore, a recent multiinstitutional study demonstrated the

reproducibility of MALDI-TOF, with good coefficients of variation justifying the use of MS for relative quantitation of oligosaccharides [88].

4.3.3. *Analysis of Derivatized Oligosaccharides*

4.3.3.1. *Methylesterification.* Methylesterification of the carboxylic group of sialic acid stabilizes the oligosaccharide and allows the simultaneous detection in the reflectron positive ion mode of neutral and sialylated oligosaccharides without metastable decay [85]. The N-glycans are dissolved in dimethylsulfoxide and methyliodide, the samples are left for 2 hours at room temperature, and excess reagent is evaporated. The samples and the reagents need to be perfectly anhydrous, otherwise the reaction is incomplete.

4.3.3.2. *Methylation.* Permethylation using the solid sodium hydroxide procedure permits the derivatization of sialylated and neutral oligosaccharides [89], although this procedure needs a clean up that is not compatible with a high number of samples. It is a remarkable tool for structural information [90].

MALDI-TOF analysis of the permethylated N-glycome gives the distribution of the sialylated and the neutral N-glycans.

Electrospray ionization ion trap mass spectrometry (ESI-IT-MS) allows the analysis and structural characterization of permethylated oligosaccharide in complex mixtures and has the capacity to perform multiple stages of fragmentation (MS^n). The first stage (MS^1) yields resolution of the methylated glycan mixture and the chemical composition of each isobaric oligosaccharide. The second and third stages (MS^2 and MS^3) provide information on the nature of the antennae, the branching pattern, the core structure, and the core fucosylation [83, 91].

The permethylated oligosaccharides obtained after hydrolysis, reduction, and acetylation are analyzed as their permethyl alditol acetates by gas chromatography–mass spectrometry (GC–MS). This procedure permits linkage analysis and the quantification of 2,3- and 2,6-linked sialic acid. Key features of the linkage analysis of the N-glycome are as follows: (1) the abundance of 2-linked mannose; 2,4- and 2,6-linked mannose represent the biantennary complex glycans and tri- and/or tetraantennary structures, respectively; (2) comparison of the analysis before and after desialylation, especially the 3- and 6-linked galactose decrease and the concomitant increase of terminal galactose, indicates the sialic acid position on the galactose residues; (3) 4,6-linked *N*-acetylglucosamine indicates the presence of core α1,6-fucosylation, and 3,4-linked *N*-acetylglucosamine corresponds to α3-fucosylation; and (4) 3,4,6-linked mannose indicates bisected complex structures [83, 90, 92].

4.3.3.3. *Fluorophore-Labeled N-Glycome.* The reducing terminus of N-glycans is coupled with a fluorophore that contains a primary amino group. The Schiff's base formed is stabilized by reduction with sodium cyanoborohydride to a secondary amine. The fluorophore-labeled oligosaccharides can be separated by electrophoresis (fluorophore-assisted carbohydrate electrophoresis, FACE) [93]. Adaptation of this protocol to a DNA sequencer allows high-sensitivity detection, high throughput, and reliable quantitation. The fluorophore used is 8-amino-1,3,6-pyrene trisulfonic acid (APTS); the separation of the oligosaccharide is performed either on a polyacrylamide gel-based sequencer or on multicapillary DNA sequencers [72, 94]. Compared with mass spectrometric methods, this protocol resolves isobaric glycan stereoisomers, but the possibilities of characterization of an unknown compound are limited to the use of standards for comigration and to enzymatic degradations.

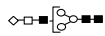
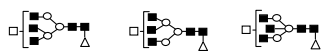
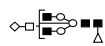
4.3.3.4. *Pyridylaminated Oligosaccharides.* Pyridylamination involves reductive amination of the reducing end of the N-glycan by coupling the sample with 2-aminopyridine (2-AP) in the presence of borane dimethylamine complex [95]. The study of human TSNG with this technique identified 31 different N-glycans [96]. But to obtain this result, it is necessary to sequentially separate the mixture of released 2-AP-coupled N-glycans by HPLC on three different columns. This protocol is unfortunately not suitable for quantitative glycan profiling and cannot be applied to a great number of samples. A recent report describes a MALDI-TOF analysis of the 2-AP derivatized and methylesterified N-glycans [97].

5. Qualitative and Quantitative Characterization of the Normal Human TSNG

Structural identification and distribution of the N-glycans comprising the human TSNG has been undertaken by various techniques, such as HPLC [96], DNA-FACE [73, 74], and MS [83, 97]. The structures identified are schematized in Table 1.

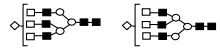
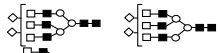
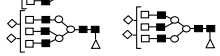
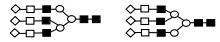
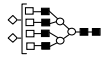
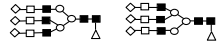
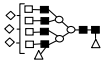
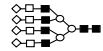
A MALDI-TOF mass spectrum of a methyl-esterified N-glycome of a normal subject is characterized by the presence of 33 ions corresponding to different isobaric N-glycans (Fig. 4A). Traces of oligomannosidic structures are found (structures 1 and 4 in the Fig. 4A) but the majority corresponds to complex-type N-glycans. The most intense ions at *m/z* 1969 and 2274 correspond to the mono- and disialylated biantennary "complex" structures (structures 20 and 27 in Fig. 4A and Table 1). The ratio of bi- to triantennary structure is 5 (i.e., structures 27 vs 38) as well as the ratio of tri- to tetraantennary (i.e., structures 38 vs 44). The MALDI-TOF analysis does not

TABLE 1
STRUCTURE OF N-LINKED GLYCANS FROM HUMAN TSNG

N°	*m/z*	Chemical composition	Structure[a]
1	1257	$Hex_5HexNAc_2$	
2	1283	$Hex_3HexNAc_3DeoxyHex$	
3	1299	$Hex_4HexNAc_3$	
4	1419	$Hex_6HexNAc_2$	
5	1486	$Hex_3HexNAc_4DeoxyHex$	
6	1502	$Hex_4HexNAc_4$	
7	1543	$Hex_3HexNAc_5$	
8	1582	$Hex_7HexNAc_2$	
9	1604	NeuAcMe $Hex_4HexNAc_4$	
10	1648	$Hex_4HexNAc_4DeoxyHex$	
11	1664	$Hex_5HexNAc_4$	
12	1689	$Hex_3HexNAc_5DeoxyHex$	
13	1705	$Hex_4 HexNAc_5$	
14	1743	$Hex_8HexNAc_2$	
15	1807	NeuAcMe $Hex_4 HexNAc_4$	
16	1810	$Hex_5HexNAc_4DeoxyHex$	
17	1851	$Hex_4HexNAc_5DeoxyHex$	
18	1867	$Hex_5HexNAc_5$	
19	1953	NeuAcMe $Hex_4 HexNAc_4DeoxyHex$	
20	1969	NeuAcMe $Hex_5 HexNAc_4$	
21	2010	NeuAcMe $Hex_4 HexNAc_6$	
22	2013	$Hex_5HexNAc_5DeoxyHex$	
23	2029	$Hex_6 HexNAc_5$	

(*continues*)

Table 1 (*Continued*)

N°	*m/z*	Chemical composition	Structure[a]
24	2115	$NeuAcMeHex_5HexNAc_4DeoxyHex$	
25	2172	$NeuAcMeHex_5HexNAc_5$	
26	2175	$Hex_6HexNAc_5DeoxyHex$	
27	2274	$NeuAcMe_2\ Hex_5\ HexNAc_4$	
28	2318	$NeuAcMeHex_5HexNAc_5DeoxyHex$	
29	2334	$NeuAcMe\ Hex_6HexNAc_5$	
30	2394	$Hex_7HexNAc_6$	
31	2420	$NeuAcMe_2Hex_5HexNAc_4DeoxyHex$	
32	2539	$Hex_7HexNAc_6DeoxyHex$	
33	2623	$NeuAcMe_2Hex_5HexNAc_5DeoxyHex$	
34	2639	$NeuAcMe_2Hex_6\ HexNAc_5$	
35	2685	$Hex_7\ HexNAc_6DeoxyHex_2$	
36	2699	$NeuAcMeHex_7HexNAc_6$	
37	2785	$NeuAcMe_2Hex_6HexNAc_5DeoxyHex$	
38	2944	$NeuAcMe_3Hex_6HexNAc_5$	
39	3004	$NeuAcMe_2Hex_7HexNAc_6$	
40	3090	$NeuAcMe_3Hex_6HexNAc_5DeoxyHex$	
41	3309	$NeuAcMe_3Hex_7HexNAc_6$	
42	3455	$NeuAcMe_3Hex_7HexNAc_6DeoxyHex$	
43	3601	$NeuAcMe_3Hex_7HexNAc_6DeoxyHex_2$	
44	3614	$NeuAcMe_4Hex_7HexNAc_6$	
45	3760	$NeuAcMe_4Hex_7HexNAc_6DeoxyHex$	
46	3906	$NeuAcMe_4Hex_7HexNAc_6DeoxyHex_2$	

m/z values refer to $[M+Na]^+$ ions of methylesterified N-linked glycan.

[a] Symbols for the structural formulas are defined as follows: solid square, GlcNAc; open square, Gal; open circle, Man; triangle, fuc; open diamond, *N*-acetylneuraminic acid.

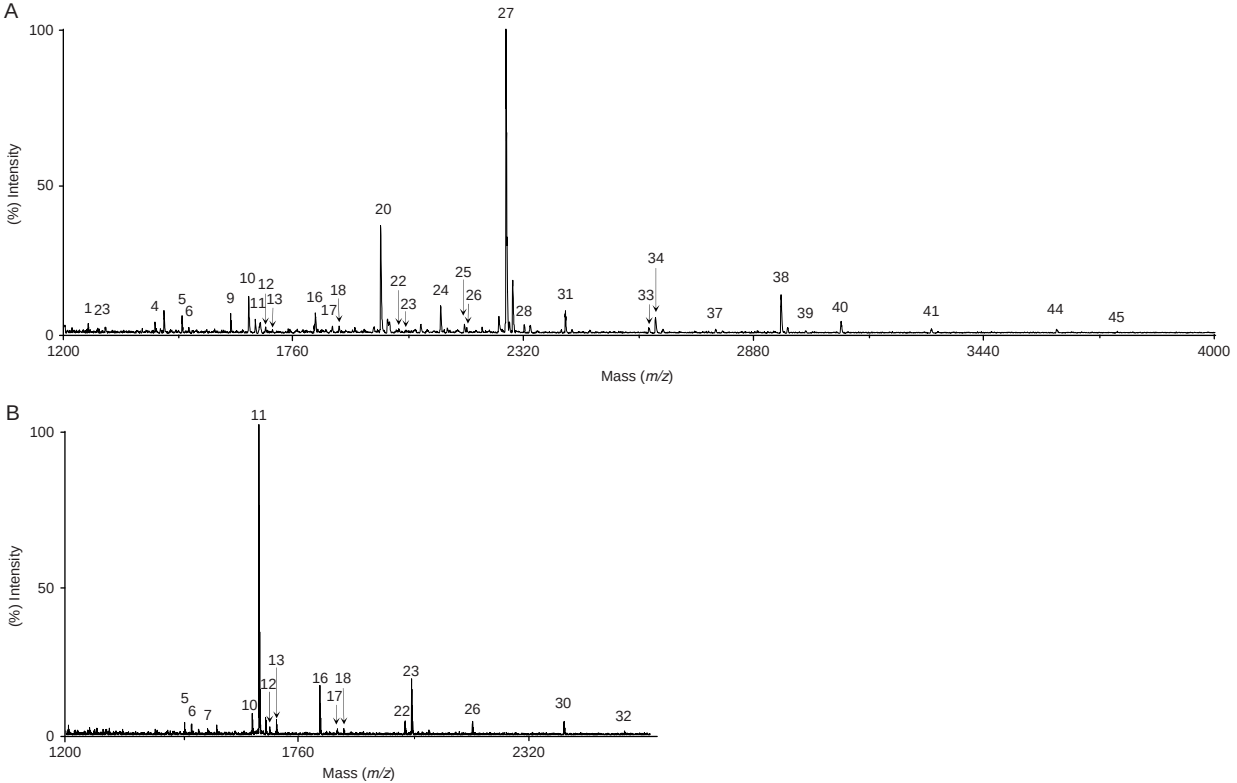


FIG. 4. MALDI-TOF-MS analysis of human TSNG. Spectra obtained from (A) Methyl-esterified N-linked oligosaccharides from normal subject and (B) desialylated N-linked oligosaccharides from normal subject. The numbers correspond to the structures in Table 1.

reflect the extreme diversity of the structures present since an identical ion could correspond to different isobaric structures. Three types of diversity cannot be characterized: (1) the branching pattern of the GlcNAc residues on the pentasaccharidic core, (2) the position of the fucose α1,6- or α1,3-linked, and (3) the anomery of the linkage of the sialic acid α2,3- or α2,6-linked on the galactose.

Analysis of the permethyl alditol acetates by GC–MS gives an indication of the position of the fucose residue which is principally α1,6-linked to the core GlcNAc residue, and only traces of α1,3-linked fucose are detected. α2,6-linked *N*-acetylneuraminic acid predominates; the ratio of α2,6 to α2,3 is approximately 5. Trace amounts of neutral oligosaccharides are observed corresponding to agalactosylated GlcNAc terminated bi- or triantennary complex-type N-glycans (i.e., structures 5 and 12 in Table 1) and to the corresponding monogalactosylated oligosaccharides (i.e., structures 10 and 17 in Table 1). Glycans possessing a bisecting GlcNAc residue are present as very minor components (structure 33 in Table 1 and Fig. 4A) as demonstrated by the presence of traces of 3,4,6-linked mannose in the GC–MS analysis [83]. These 33 isobaric structures correspond to approximately hundred different glycans.

After desialylation of the N-glycome the spectrum is simpler and confirms the previous observation on the branching and fucosylation pattern (Fig. 4B). Approximately 18% of structures are fucosylated and 6% are bisected.

Most of the techniques give relative quantification to either an internal standard or to the most intense peak of the spectrum or of the diagram. Recently, absolute concentration was achieved by HPLC analysis of 2-AP-coupled N-glycans [97]. Quantification of the oligosaccharide is not only representative of glycosylation variations, but also reflects the variation of individual glycoprotein concentrations and the hydration of the serum; for these reasons, it is preferable to express glycosylation differences with intensity ratios of different oligosaccharide (i.e., the ratio of intensities of a biantennary complex oligosaccharide to the corresponding triantennary glycan). Another limitation encountered by quantification is the high variation coefficient (CV) of low-intensity signals.

The variation of distribution of the N-glycans can be defined by calculating the ratio of signal intensities between different glycans. Recently, Callewaert *et al.* have defined a biomarker, the GlycoCirrhoTest, that distinguishes compensated cirrhotic from non-cirrhotic chronic liver disease patient and which is obtained by calculation of the logarithm of the concentration (or of the ions intensities) ratio of structures 22 and 23 (Table 1) obtained from a desialylated N-glycome [74].

6. Physiological Variations of the N-Glycome

Variations of the TSNG according to age and gender have not yet been studied, but previous studies indicate the possible physiological variations [61, 98].

6.1. Ageing

Modification of galactosylation of IgG N-glycans has been described with ageing. For example, an increase of galactosylation from birth to a maximum at approximately 25 years and then a decrease with ageing was observed by Parekh [98]. Other glycoprotein glycosylation might vary with age also.

6.2. Pregnancy and Estrogen

Estrogen induces an effect on the degree of N-glycan branching on transferrin [45] and on AGP [61]. A decrease in fucosylation and in sialyl-Lewis^x expression has been demonstrated during estrogen treatment [61].

An example of a TSNG of a second trimester of pregnancy is presented in Fig. 5. An increase in the relative intensity of ions corresponding to the triantennary structure 23 and a decrease of the relative intensity of ions corresponding to the fucosylated structures 16 and 26 can be observed.

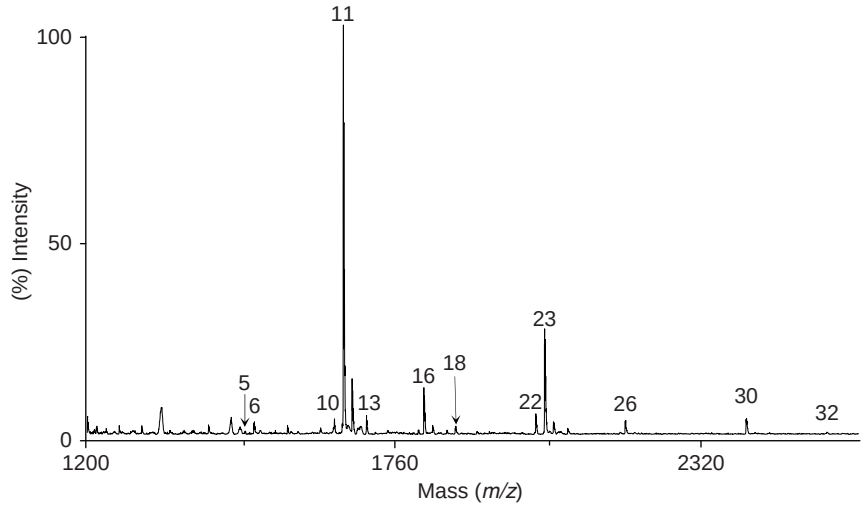


Fig. 5. MALDI-TOF-MS analysis of human TSNG. Spectra obtained from a desialylated N-linked oligosaccharides from a second trimester pregnancy. The numbers correspond to the structures in Table 1.

7. Acquired Modifications of the TSNG

7.1. Liver Fibrosis and Cirrhosis

Many modifications in *N*-glycosylation have been demonstrated in hepatic cirrhosis [99–101]. Modifications of the TSNG during liver fibrosis and cirrhosis were first demonstrated with a DNA sequencer of desialylated N-glycans, an increase of structures containing bisecting GlcNAc being demonstrated [74].

Morelle *et al*. elucidated the modifications of the N-glycan mix constituting the TSNG in patients with cirrhosis by mass spectrometric analysis of desialylated or methylesterified oligosaccharides and using permethylation they characterized three types of modifications: (1) the presence of an important population of N-glycans with a bisected GlcNAc (structures 7, 12, 13, 22, 25, 28, and 33 in Fig. 6A; and structures 7, 12, 13 and 22 in Fig. 6B); (2) the relative increase in α1,6-fucosylated oligosaccharides (structures 5, 16, 22, and 26 in the desialylated N-glycome, shown in Fig. 6B); and (3) the presence of neutral agalactosylated oligosaccharides (structures 5, 10, 12, 16, 17, and 22 in Fig. 6A and Table 1) [83].

Callewaert *et al.* have defined and shown the efficiency of a new cirrhosis marker, the GlycoCirrhoTest (AUC = 0.87, specificity = 100%; sensitivity = 75%), but this marker was not relevant for less-advanced fibrosis stages (from F0/F1 to F3 according to Metavir system) [102]. One of the reasons of the lack of sensitivity is probably the small number of oligosaccharide markers that were studied. Higher resolution techniques and also allowing the simultaneous study of the sialylated and the neutral N-glycans could permit the discovery of novel markers of fibrosis. In a recent study, with the use of surface-enhanced laser desorption ionization time-of-flight (SELDI-TOF) Kam *et al*. found four ions at *m/z* values corresponding to ions showing positive correlations with fibrosis stages. From these values the authors have predicted novel structures of N-glycans not found previously in the TSNG, but this data need to be confirmed by more specific techniques (enzymatic degradations, methylation analysis) [82].

Another possible reason for the poor sensitivity of fibrosis detection is the superposition of different glycoprotein glycosylation modifications. For example, the neutral and most of the bisected GlcNAc oligosaccharides are present on pro-inflammatory galactose-deficient immunoglobulins, but the same glycans might be present on fibrotic liver-synthesized glycoproteins and may be only the latter ones would be a marker of the liver disease.

The N-glycome described in Fig. 6 was obtained from a patient suffering from liver fibrosis with acute alcoholic hepatitis. It is characterized by the presence of a relative increase of multiantennary difucosylated oligosaccharides

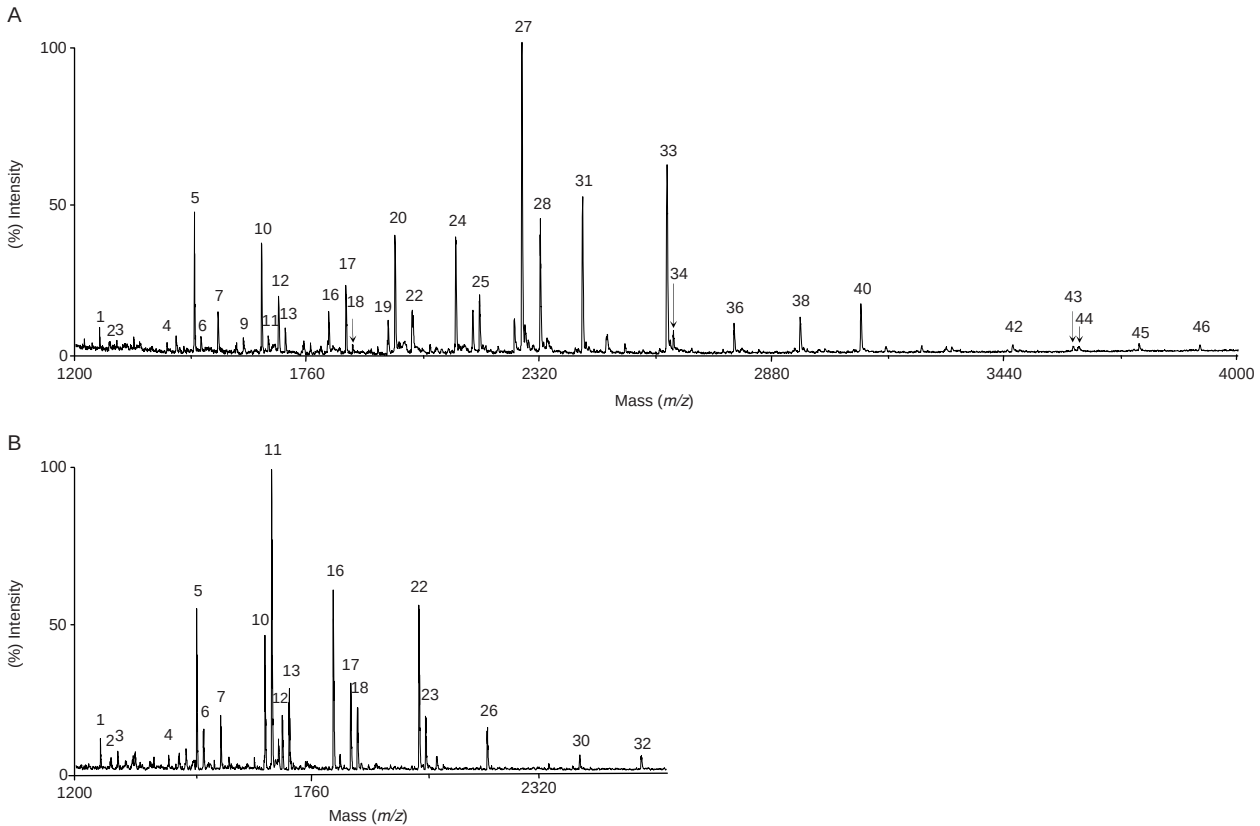


FIG. 6. MALDI-TOF-MS analysis of thuman TSNG. Spectra obtained from (A) Methyl-esterified N-linked oligosaccharides from a cirrhotic patient and (B) desialylated N-linked oligosaccharides from a cirrhotic patient. The numbers correspond to the structures in Table 1.

(structures 43, 44, and 45 in Table 1); these oligosaccharides might relate to the inflammation and to glycosylation modifications of AGP.

7.2. Inflammatory Disorders

During inflammatory disorders, systemic changes have been referred to acute phase response. Among this response, modifications of serum protein concentrations occur either as an increase of positive acute phase proteins or as a decrease of negative acute phase proteins. Most of the major serum N-glycosylproteins are acute phase proteins [i.e., positive: complement system, antiproteases (α1-antitrypsin, α1-antichymotrypsin), haptoglobin, AGP; negative: transferrin]. The various etiologies that lead to substantial changes in the serum concentration of acute phase proteins include infection, trauma, surgery, burns, tissue infarction, various immunologically mediated and crystal-mediated inflammatory conditions, and advanced cancers [58, 103]. As a consequence, in all these conditions, the TSNG should be modified.

During inflammation, the glycosylation modifications are not only quantitative but also qualitative.

7.2.1. *Modifications of Glycosylation During Acute Inflammation*

A transient decrease in the branching of N-glycan has been described on AGP, accompanied by an increase in highly fucosylated glycoforms [58, 59].

7.2.2. *Modifications of Glycosylation During Chronic Inflammation*

Chronic inflammation is correlated with modifications of the N-glycan branching, with an increase in the tri- and tetraantennary oligosaccharides, and also with an increase in fucosylation. This has been observed in numerous acute phase proteins: AGP [59, 104], haptoglobin [105], and α1-antichymotrypsin [106]. The fucose residue in these cases is α1,3-linked, part of a sialyl-Lewis^x epitope and acts as a counterreceptor, inhibitor of selectin-mediated leucocyte–endothelium interactions modulating the extravasations of leukocytes [104].

7.3. Cancer

Numerous alterations of *N*-glycosylation have been described in cancers. Three types of N-glycome modifications can be encountered: (1) the inflammation induced by the cancer modifies the concentration and the glycosylation of the acute phase proteins, (2) presence in the TSNG of cancer cell glycans with a primary structure preexisting on serum glycoproteins, and

(3) presence in the TSNG of cancer cells N-glycans with a primary structure different from serum glycoprotein oligosaccharide.

Kyselova *et al.* [80] have described the modification of the TSNG during metastatic prostate cancer. Their results demonstrate the presence of 12 N-glycans, out of which 6 were fucosylated, that the authors considered as cancer-specific N-glycans and potential prostate cancer biomarkers. Among these glycans, seven tri- or tetraantennary glycans had an increased relative intensity. A diminished relative intensity was found for the disialylated biantennary oligosaccharide. These results should be also linked to the modifications of AGP and haptoglobin during advanced malignancies, where increased fucosylation, branching, and sialylation are found [65, 107, 108]. Another modification described by Kyselova *et al.* is the increase of the relative intensity of structure 5 (Table 1). This oligosaccharide is typical of IgG, and the increase during prostate cancer of agalactosylated IgG, which has been previously described, would explain its increased presence in the TSNG [27].

7.4. Immunoglobulin Glycosylation Modifications

The glycosylation pattern of IgG, biantennary neutral N-glycans bisected or not, distributed into sialylated oligosaccharides and three types of neutral oligosaccharides (G_0, G_1, and G_2) are modified during numerous pathological processes [11, 21–27] with implications in the inflammatory response. The follow-up and the prevention of the complications of these diseases with the TSNG will be possible as the characteristic agalactosylated glycans (i.e., structures 5 and 10 in Table 1) can easily and specifically be identified and quantified in the spectrum.

8. Congenital Modifications of the TSNG

CDG are a family of genetic diseases with a broad clinical presentation resulting from defects in the synthesis of glycans [3]. Twenty disorders of *N*-glycosylation have been reported, out of which four are combined *O*- and *N*-glycosylation defects. CDG type I results from a defect in the assembly of the dolichol-phosphate-linked oligosaccharide precursor, and CDG type II results from a defect in the processing of the glycan in ER and the Golgi.

The analysis of the TSNG of 24 subjects suffering of CDG type I indicated an increase of the α1,6-fucosylation of the biantennary structures and a significant decrease of the triantennary glycans. The primary protein glycosylation abnormality, in CDG type I, is the partial absence of N-glycans on

glycoproteins. Transferrin, lacking one or two chains, is easily detected by isoelectrofocusing and this test is the most widely used for the disease. Since the TSNG does not allow the measurement of a defect of glycosylation, this abnormality is not seen. The TSNG abnormality observed in CDG is probably related to an altered liver function, as seen in liver fibrosis [73]. Increased fucosylation and expression of sialyl-Lewis^x determinant has been described on AGP, α1-antitrypsin, and α1-anti-chymotrypsin of patient with CDG type I. The observed increase of α1,3-fucosylation is induced by a chronic hepatic inflammatory response [109]. Increased branching of N-linked glycans of transferrin and α1-antitrypsin was demonstrated by Mills *et al.* [54]. The overall N-glycan pattern, observed in TSNG, indicates a decreased branching. Therefore, some glycoproteins react differently to the *N*-glycosylation defect [73] and the TSNG is the sum of all individual glycoprotein N-glycome and of the various superposed physiological alterations (genetic defect with secondary inflammation and liver fibrosis).

In CDG type II, the processing defect of *N*-glycosylation modifies the TSNG qualitatively. In CDG type IIa, the deficit in GlcNAc-TII results in the appearance of characteristic hybrid-type N-glycans [13, 110]. Faid *et al.* have described a typical methylated TSNG of a patient suffering from CDG type IIa, the TSNG of this patient presents a major hybrid structure (9 in Table 1) characteristic of the absence of GlcNAc TII [111]. In the same report, the authors described a second patient with a mild form of CDG type II due to a deficiency of the Cog1 subunit complex [112]. The TSNG presented a characteristic pattern with a different distribution of the oligosaccharides with an increase of structures lacking sialic acid and galactose residues (structures 15, 20 24, 29, and 34 in Table 1) [112] at the difference of the first patient, and in the second case, the TSNG modifications are less specific and the precise alteration of the N-glycan metabolism cannot be determined.

The desialylated TSNG of a patient suffering from CDG type IIx was shown by Sagi *et al.* [79]. Although the authors demonstrate the efficiency of the technique for the diagnosis of the CDG, with the presence of truncated oligosaccharides in the spectrum (structures 5 and 10), it should be noted that the TSNG is similar to the one obtained in a cirrhotic patient, and there would not be a difference between this CDG type IIx and a CDG type I with liver fibrosis; therefore, the use of TSNG for a specific diagnostic of CDG has limitations.

Other congenital defects such as galactosemia [113], hereditary fructose intolerance [53], and cystic fibrosis [114] have been shown to modify *N*-glycosylation, and therefore in these diseases the TSNG might be altered consequently.

9. Conclusions

The study of the TSNG is a new field in clinical chemistry. Preliminary studies have demonstrated the interest of this analysis in the domain of CDG and hepatic cirrhosis. The promises of this analysis in the exploration of inflammatory diseases, autoimmune diseases, and cancers need to be demonstrated on large groups of patients, especially concerning the sensitivity and the specificity of the glycan variations. The understanding of the mechanisms of TSNG variations will need complementary analytical explorations to define on which glycoprotein these occur. The knowledge of individual glycoprotein N-glycome variations will then allow the use of the TSNG as a tool for diagnosis and prognosis of diseases. The increase in the sensitivity of the mass spectrometer will soon authorize the analysis of depleted serum, after removal of the 10–15 high-abundant serum glycoproteins; such possibility may permit the detection of low-abundance N-linked oligosaccharides that may be putative disease biomarkers [115, 116]. The TSNG is only a fraction of the human glycome; the exploration of other glycomes such as urinary N-glycome, urinary free oligosaccharides [117], serum glycolipids [118], serum *O*-glycosylation [111, 119], spinal fluid glycome is in progress. The new biomarkers defined by the TSNG will probably be discovered; their use in the future with the progress in analytical tools and automation in the glycome field will expand and certainly become indispensable to the clinician.

Acknowledgment

The author thanks Dr. D. Allorge, Dr. C. Carnoy, Dr. C. Flahaut, and Professor Ph. Roussel for helpful discussions, and for critically reading the manuscript.

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NUTRITIONAL BIOCHEMISTRY OF SPACEFLIGHT

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Abbreviations

EPA	eicosapentaenoic acid
EVA	extravehicular activity
ISS	International Space Station
PTH	parathyroid hormone
RBC	red blood cell
WHO	World Health Organization

0065-2423/08 $35.00
DOI: 10.1016/S0065-2423(08)00403-4

1. Abstract

As we approach the end of the first 50 years of human space travel, much has been learned about adaptation to microgravity and the risks associated with extended-duration space exploration. As the frequency and duration of flights grew, nutrition issues became more critical and the questions to be answered became more complex: What are the nutrient requirements for space travelers? Can nutrients be used as tools to mitigate the negative effects of space travel on humans? How does nutrition interrelate with other physiological systems (such as muscle, bone, and cardiovascular system) and their adaptation to microgravity? Much research has been done over the decades in both actual spaceflight and ground-based analogs. We review here much of what is known, and highlight areas of ongoing research and concerns for future exploration of the Moon, Mars, and beyond.

2. Introduction

Nutrition has proven critical to the success of exploration journeys throughout history, and space exploration will be no different. In the five decades since humans first orbited the planet, much has been learned about human adaptation to microgravity. Short- (days to weeks) and long-duration (4–6 months) stays in space have been relatively common, with a very small number of missions to date extending beyond 6 months. As the United States looks to begin the next phase of exploration with a return to the Moon, questions about the effects of partial gravity (such as 1/6-gravity on the Moon), radiation, lunar dust, and other factors are being honed. Questions associated with much longer (2- to 3-year round-trip) journeys to other planets are also being posed and answers are being actively pursued.

As with nutrition in the terrestrial environment, nutrition issues of spaceflight tend to focus on nutrient requirements for optimal health and the ability of nutrition to mitigate disease. Unique aspects of nutrition during space travel include the overarching physiological adaptation to weightlessness, psychological adaptation to extreme and remote environments, and the ability of nutrition and nutrients to serve as countermeasures to ameliorate the negative effects of spaceflight on the human body. Accordingly, defining the nutrient requirements for spaceflight and ensuring provision and intake of those nutrients are primary issues for crew health and mission success. Additionally, as countermeasures (such as exercise and pharmacological agents) for other systems are sought, care needs to be taken that they do not have negative influences on nontarget systems.

Despite the fact that a relatively small number of individuals have traveled in space (<500 total to date), many studies have been conducted in an attempt to characterize human adaptation to microgravity. Many of these involve evaluations of crew members before and after spaceflight, but a limited number have been conducted during flight as well. The Space Shuttle has provided a platform for short-duration studies, while the US Skylab, Russian Salyut and Mir space stations, and the International Space Station (ISS) have provided for longer missions. Given the limited number of flights, and challenges associated with in-flight sample and data collection, ground-based analogs have been utilized to simulate different aspects of weightlessness. The most common human analog is head-down-tilt bed rest [1], with Antarctic, undersea, and other analogs also serving in this important role [2]. Animal and cell models have also been used to expand our understanding of physiological changes, in-flight studies, as well as ground-based analogs (such as rodent hindlimb suspension).

We review here the general effects of space travel on human nutrition and on physiological systems with nutritional relevance, including both spaceflight and ground-based analog studies. We also discuss areas where nutrition may serve as a countermeasure to help mitigate the negative effects of spaceflight on human physiology, and the issues of future exploration missions.

3. Nutrition

3.1. Food Systems

Ensuring that the spacecraft food systems provide palatable, safe, and nutritious foods is obviously critical for any space mission. For the early space programs (Mercury, Gemini, Apollo, and even the Space Shuttle), with mission durations from hours to a week or two, food provision was critical, but for missions of such short duration (similar to a short camping trip), understanding specific spaceflight nutrient requirements was not mandatory [3–6]. However, as mission durations increased from weeks to months, as on space stations (Skylab, Mir, ISS), the risks associated with potential deficiencies, or even toxicities, increased as well. The longer space station missions have included semi-closed food systems, with periodic resupply and transient exposure to unique and fresh foods [5–8]. Exploration missions will have a more closed food system, with the potential for supplementation with food sources grown *in situ* [5–7].

From the early days of the space program [9–11], development of foods for spaceflight has proven a significant challenge, yet the design criteria have

changed little since then: minimal crumbling, ease of preparation and consumption in microgravity, minimal trash volume, high palatability. With one exception, the food systems used in every space program to date have been entirely shelf-stable, and they are composed primarily of rehydratable or thermostabilized food items [6, 7]. Although these foods are known to have lower hedonistic value (palatability) than fresh or frozen foods, ground-based studies have clearly shown that the Shuttle food system can adequately support most nutritional requirements [12]. Skylab is the only US program that has included frozen foods [6, 7]. Nutrient requirements have been defined for extended-duration ISS missions [13, 14], and with a few exceptions (most notably vitamin D insufficiency, and iron and sodium excess), the actual menus meet these requirements (Table 1). As discussed below, vitamin D supplements are provided to mitigate the dietary insufficiency.

It is imperative that adequate resources be provided to support food consumption. A reliable food system must include a variety of palatable foods and the means to process them (such as rehydration, heating, and cooling). Time (for meal preparation, consumption, and clean-up) is another limited resource that often hinders dietary intake, especially on shorter Shuttle missions.

The original plans for the ISS food system included the use of freezers and refrigerators for food storage and preparation. This would have provided a more palatable food system, but difficult decisions had to be made regarding power, mass, volume, and cost, and ultimately the use of freezers and refrigerators for food was dropped from the plans. It is difficult to balance the intangible potential increase in dietary intake and psychological support against tangible dollar and power allocations, both of which are typically constrained.

3.2. Energy Intake

Despite indications that in-flight and preflight energy requirements are similar [15, 16], energy intake during flight is commonly less than before flight [8, 15, 17–26]. From the Apollo program through the more recent flights, crew member dietary intakes have averaged about 70% of predicted requirements [20]. Exceptions do exist, and a number of ISS crew members have been able to consume recommended dietary intake requirements and maintain body mass [20]. In other cases, metabolic experiments have required subjects to consume a eucaloric diet, such as during Skylab [27] and European flights to the Mir space station [28]. These were successful, further documenting that nominal dietary intake is achievable. The obvious concern about reduced dietary intake is the risk of body mass loss and dehydration [29],

TABLE 1
PLANNED (MENU) AND TARGETED NUTRIENT INTAKE ON INTERNATIONAL SPACE STATION MISSIONS

	Menu content[a]	NASA spaceflight requirement
Energy (kcal/day)	2903 ± 168[b]	Based on WHO [16]
Energy (% WHO)	99 ± 13	–
Total carbohydrate (% of kcal)	50 ± 3	50 – 55
Total protein (% of kcal)	17 ± 1	12 – 15
Animal protein (g/day)	72 ± 7	60%
Vegetable protein (g/day)	33 ± 3	40%
Total fat (% of kcal)	31 ± 1	30 – 35
Total dietary fiber (g/day)	33 ± 5	10 – 25
Retinol equivalents (μg/day)	1446 ± 213	1000 μg retinol equivalents
Vitamin D (μg/day)	4.3 ± 1.1	10
Vitamin E (total α-tocopherol equivalents) (mg/day)	12.5 ± 1.7	20
Vitamin K (phylloquinone) (μg/day)	108 ± 18	80
Vitamin C (ascorbic acid) (mg/day)	191 ± 42	100
Thiamin (mg/day)	2.0 ± 0.1	1.5
Riboflavin (mg/day)	2.2 ± 0.2	2.0
Niacin (mg/day)	29.7 ± 1.9	20 mg niacin equivalents
Pantothenic acid (mg/day)	5.3 ± 0.5	5.0
Vitamin B_6 (mg/day)	2.3 ± 0.2	2.0
Total folate (μg/day)	444 ± 48	400
Vitamin B_{12} (cobalamin) (μg/day)	4.6 ± 0.7	2.0
Calcium (mg/day)	1016 ± 117	1000 – 1200
Phosphorus (mg/day)	1864 ± 179	1000 – 1200 (not to exceed 1.5 times calcium)
Magnesium (mg/day)	430 ± 41	350
Iron (mg/day)	23 ± 5	10
Copper (mg/day)	3.7 ± 1.0	1.5 – 3.0
Zinc (mg/day)	23 ± 7	15
Manganese (mg/day)	5.8 ± 0.7	2 – 5
Selenium (μg/day)	145 ± 16	70
Iodine (mg/day)	1.1 ± 3.0	0.15
Sodium (mg/day)	5624 ± 578	$<$ 3500 mg
Potassium (mg/day)	4044 ± 368	3500 mg
Water (g/day)	2212 ± 175	1 ml/kcal, no less than 2 liters per day

[a] Menu data are derived from either proximate analysis of space foods (macronutrients, most minerals) or estimations (animal protein, vegetable protein, all vitamins, selenium) from similar items in the Nutrition Data System for Research (NDS-R) database, versions 4.03/31, 4.05/33, 4.06/34, 5.0/35, 2005, and 2006, developed by the Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN [303].

[b] All data are mean $\pm$ SD, and represent the average from menus of 16 ISS astronauts.

but existing data suggest that many systems are affected by inadequate nutrient intake, including the muscle, bone, cardiovascular, immune, and other systems.

The cause of reduced dietary intake during flight is generally unknown [30]. Food palatability is occasionally reported as a cause of reduced in-flight intake, and many anecdotal reports exist of changes in taste and aroma of food during flight. One hypothesis is that fluid shifts and congestion associated with microgravity (especially during the first few days) can alter taste and odor perception. However, experimental research has not been able to clearly document changes in taste or olfaction [31, 32].

A common cause of reduced dietary intake during the first days of a mission is space motion sickness [33]. Its effects typically pass after the first several days of flight, but the inadequate dietary intake often extends well beyond the first week [14]. Other flight-related changes in gastrointestinal function may occur. Fluid shifts, in combination with reduced fluid intake, would tend to decrease gastrointestinal motility and increase transit time [34]. It has been hypothesized that other gastrointestinal functions may be altered in space, including microflora production of vitamin K, but few or no data are available to support this.

Skylab crew members ate essentially 100% of their recommended [16] energy requirements [14]. Although the Skylab crews were involved in metabolic studies that required complete dietary intake [35], this result demonstrates that when required to, astronauts can consume the recommended amounts of food during spaceflight. Thus, hypotheses regarding inability to consume the requisite amount of food because of stomach fullness or other factors are not likely to fully explain decreased in-flight dietary intake. It is difficult to determine if the intakes on Skylab were related more to the requirement to consume the food or to the fact that the food was more palatable because of the additional variety of frozen foods available; however, it is difficult to argue that increased palatability is not beneficial.

The gap between energy intake and expenditure is further widened by increased amounts of exercise during flight associated with physical countermeasures to spaceflight effects. Results of metabolic experiments on the US Skylab missions showed that ingestion of the prescribed amount of calories did not maintain body mass [27], and it is clear that inadequate energy intake will ensure loss of body mass. Furthermore, inadequate energy intake is associated not only with loss of fat tissue, but also with decreased protein synthesis [36] (in-flight models), increased protein catabolism [37] (in ground-based models), and subsequent loss of lean tissue mass.

3.3. Nutritional Status Assessment

Clinical assessment of flight crews before and immediately after flight has been standard medical practice from the first flights. In some cases, the markers that were assessed reflected nutritional status as well as other clinical

measures to assess overall health status, but only in the mid-1990s was a dedicated, comprehensive clinical nutritional assessment profile developed and implemented [18, 20, 38]. The primary goal was to develop a comprehensive evaluation of nutritional status that would be used to ensure optimal status before flight, to track dietary intake and nutritional status as well as possible during flight, and to evaluate crew members as soon as possible after flight to speed the return of any decrements to preflight status. Because the risks associated with suboptimal nutrition are greater on longer flights, this comprehensive evaluation was implemented only on long-duration (ISS) flights. The protocol was developed by a team of NASA intramural and extramural experts [38], and was tested provisionally with two astronauts on the Russian space station Mir as well as in a 90-day ground-based analog [18]. The protocol was determined to be a "medical requirement" for ISS missions, and has been implemented on all ISS flights since they began in 2000. The consensus protocol was reviewed at the outset (and several times over the years) by intramural, extramural, and international review panels. An evaluation of results from the crews of the first nine expeditions was published in 2005 [18]. The primary focus of the protocol is on the biochemical assessment of nutritional status, but body weight and composition are also determined, and dietary intake is monitored during flight using a food frequency questionnaire [39].

3.3.1. *Biochemical Assessment*

Twice before and once after flight, fasting blood samples and two 24-hour urine samples are collected for a broad range of analyses, including protein, minerals, vitamins, antioxidants and oxidative damage markers, hematology, and general chemistry [18, 20, 38]. The biochemical markers relate not only to nutrient categories, but also to the physiological systems, including muscle and bone, that use or produce the markers, or in which the markers are associated with a risk, such as the risk of renal stone formation.

3.3.1.1. *Vitamins*

3.3.1.1.1. *Water-soluble vitamins.* Water-soluble vitamins are a key concern for space travelers, given the limited endogenous storage of many of these nutrients. They must be replenished from food that may have been stored for a long time (9–18 months) under suboptimal conditions, including the space radiation environment.

It is evident from previous long-duration spaceflight research (4–6 months) that folate status decreases after spaceflight (Fig. 1) [20]. The food system includes foods with adequate amounts of folate (Table 1), and it is unknown at this point if the decline in folate status is related to the stability of folate in food items stored during flight or if the body's requirement for folate is increased during flight, which could be related to alterations in absorption,

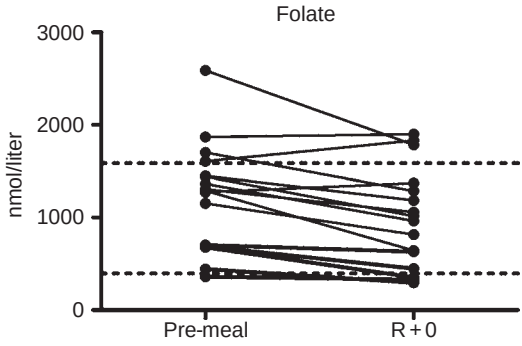

FIG. 1. Serum folate concentrations before and after 4- to 6-month spaceflights on the International Space Station. Each line represents one crew member. The "Pre-Mean" point is the average of data collected about 6 months and 6 weeks before launch. R + 0 = Recovery plus zero days, that is, landing day. These samples are typically collected 2–8 hours after landing.

metabolism, and/or excretion. Studies are underway to better understand stability issues, as well as to better characterize the time course of physiological changes during flight.

Concerns about the status of many water-soluble vitamins (and other nutrients) during spaceflight are related to their stability in the face of radiation exposure. Most vitamin B_6 in the body is found in muscle tissue, and thus the loss of muscle mass and strength related to spaceflight may also reduce the amount of the vitamin that is stored (see Section 4.2). Furthermore, in Earth-based populations, status of vitamin C, riboflavin, and other nutrients [40–43] has been related to cataract incidence, which is higher in space travelers than in the general population [44–46]. Vitamins and other antioxidants have been proposed as potential countermeasures to reduce tissue damage from space radiation, including cataracts [45, 47], but defining the required dose(s), mixtures, interactions, and/or side effects will prove a significant challenge, as it has on Earth [48–51].

Although specific concerns regarding their status during spaceflight have not been raised about other water-soluble vitamins (such as vitamin B_{12}, thiamin, niacin, biotin, pantothenate), the semi- or fully closed food systems for exploration missions raise concerns about sufficiency of these limited menus for extended durations. Furthermore, if pharmacological countermeasures are used to mitigate the effects of weightlessness on physiological systems, the potential for drug–nutrient interactions in the closed environment of space will require special attention.

3.3.1.1.2. *Fat-soluble vitamins.* Less concern is expressed about fat-soluble vitamins than about water-soluble vitamins because the body can store larger quantities of fat-soluble vitamins, but recent findings about

previously unknown functions of some of these vitamins, as well as unique aspects of spaceflight, provide specific challenges for maintaining optimum status of these nutrients.

Vitamin D has long been known to have a role in calcium metabolism, and more recently its noncalcitropic functions have been recognized [52]. According to the results of several recent studies, functionally relevant measures indicate that the lower limit of serum 25-hydroxyvitamin D (indicator of vitamin D status) should be raised from 23 to 80 nmol/liter. The mean preflight serum 25-hydroxyvitamin D for the US ISS crew members is 62 ± 14 nmol/liter (Fig. 2). People who are normally exposed to sunlight make vitamin D in their skin, but spacecraft such as the ISS and the Space Shuttle shield crew members from ultraviolet B light, a component of sunlight that can convert 7-dehydrocholesterol to 25-hydroxyvitamin D in the skin.

A decrease in vitamin D status is perhaps one of the more striking nutritional changes that occurs during spaceflight [19, 20]. Several crew members on the Russian space station Mir had serum 25-hydroxyvitamin D concentrations that were 32–36% lower during and after long-duration (3- to 4-month) missions than before the missions [19, 53], and ISS astronauts had serum 25-hydroxyvitamin D concentrations that were typically 25–30% lower after 4- to 6-month spaceflights, despite supplementation with 400 IU/day [20]. In several ISS crew members, serum 25-hydroxyvitamin D has decreased to levels considered clinically significant [20]. Crew members on the longest Skylab mission (Skylab 4, 84 days), but not the shorter

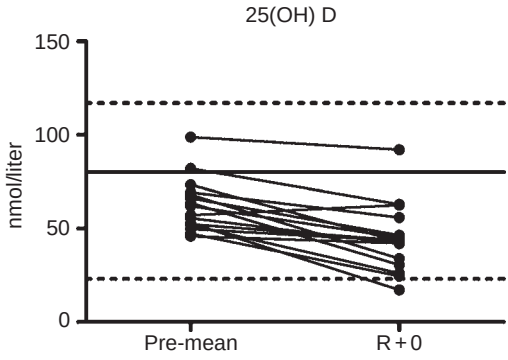

FIG. 2. Serum 25-hydroxyvitamin D [25(OH)D] concentrations before and after 4- to 6-month spaceflights on the International Space Station. Each line represents one crew member. The "Pre-Mean" point is the average of data collected about 6 months and 6 weeks before launch. R + 0 = Recovery plus zero days, that is, landing day. These samples are typically collected 2–8 hours after landing.

missions (28 and 59 days), had decreased serum 25-hydroxyvitamin D at landing despite daily vitamin D supplementation [35].

Similar decreases in vitamin D status have been found in ground-based studies of subjects living in closed-chamber facilities for extended periods [18]. Ground-based models with limited sunlight exposure are valuable for performing vitamin D supplementation trials. One of these models is the Antarctic winter, when levels of ultraviolet B radiation are essentially zero. We began a study at McMurdo Station, Antarctica, in 2007 to determine the daily dose of vitamin D needed to sustain serum levels of 25-hydroxyvitamin D during a 5- to 6-month period when there is little to no ultraviolet B exposure. It is currently recommended that ISS crew members take 800 IU/day during long-duration spaceflight.

Another important observation from the ISS nutritional status assessment was related to the relationship between parathyroid hormone (PTH) and 25-hydroxyvitamin D before and after ISS missions. Before launch, 25-hydroxyvitamin D was inversely correlated with PTH ($r = -0.72$, $P < 0.05$) (Fig. 3), but this relationship was not evident after landing, suggesting that the body's normal response to changes in vitamin D was altered [20].

The low pre- and postflight vitamin D status among crew members is an issue that needs to be resolved to redefine the appropriate amount of vitamin D to serve as a countermeasure against vitamin D deficiency in astronaut crews. This is very important for long-duration crew members, and is critical for exploration-class missions.

Vitamin K is most commonly associated with its role in blood coagulation, but more recent evidence indicates that this vitamin affects multiple physiological systems. Most notably, vitamin K is a cofactor in the

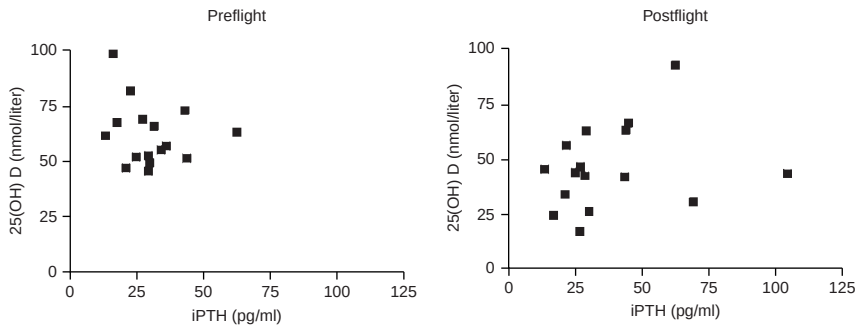

FIG. 3. Serum 25-hydroxyvitamin D [25(OH)D] and parathyroid hormone concentrations before (average of data from samples collected about 6 months and 6 weeks before launch) and after (landing day, typically collected 2–8 hours after landing) 4- to 6-month spaceflights on the International Space Station. Each symbol represents one crew member.

posttranslational synthesis of γ-carboxyglutamic acid. This amino acid is common to all vitamin K-dependent proteins, and its role is related to increasing the affinity of the proteins for calcium [54]. Data from 11 US astronauts from ISS Expeditions 1–8 (mission durations of 128–195 days during 2000–2004) revealed that on landing day serum phylloquinone (vitamin K_1) was 42% lower than it was before flight, whereas urinary γ-carboxyglutamic acid did not change [20]. Other studies have shown that vitamin K supplementation during spaceflight elevated urinary γ-carboxyglutamic acid and decreased urinary undercarboxylated osteocalcin (a bone protein), suggesting that vitamin K status is lower during spaceflight [55, 56]. The use of vitamin K as a bone loss countermeasure has been proposed and is under investigation [26].

Because oxidative stress can increase in microgravity and high-radiation environments [57–59], it may be necessary to provide enough vitamin E for astronauts' blood levels of the vitamin to be higher during spaceflight than on Earth. The antioxidant properties of vitamin E may help to counteract the free radical damage caused by high linear energy transfer radiation in space. Pretreatment with antioxidants may help decrease radiation damage during missions [60]. After learning about the promising antioxidant effects of supplemental vitamin E, many people on Earth did not hesitate to take vitamin E supplements to prevent cancer. The protective effects were not borne out in controlled studies, highlighting the difficulties of defining a specific antioxidant countermeasure for space travelers without the luxury of having data from epidemiological studies to provide an evidence base for spaceflight.

Vitamin A and β-carotene serve as biological antioxidants and have been shown in multiple studies to reduce the risk of cancer and coronary heart disease [61, 62]. Vitamin A is also directly involved in vision, gene expression, reproduction, embryonic development, and immunity, and has direct or indirect impact on the function of almost all of the body's organs. Serum levels of retinol and retinol-binding protein are significantly decreased after long-duration spaceflight [20]. As with many antioxidants, the desire to supplement with high doses in the hope of staving off one disease is high, but unwarranted and potentially counterproductive. Specifically, excess vitamin A, in levels on the order of twice the recommended daily intake, has been shown to increase bone resorption [63–66].

3.3.1.2. *Minerals.* Calcium has been one of the most studied nutrients in space travelers, solely because of its relationship with bone loss and the risk of renal stone formation. Negative calcium balance was observed during Skylab [35, 67] and Mir [19, 68] missions, with urinary and fecal calcium excretion accounting for most of the deficit [19, 35, 67, 69, 70]. Complete calcium balance studies during long-duration Skylab missions and tracer

kinetic studies during Mir missions yielded similar estimates of a loss of 200–300 mg of calcium per day from bone [19, 53, 67, 71].

Phosphorus has not been studied nearly as much as calcium, despite its relationships with calcium and bone, among other critical functions. Excretion of phosphorus after spaceflight is significantly and consistently lower than preflight excretion [20]. The causes and implications of this are currently being evaluated in both ground-based and flight studies.

Magnesium is required as a cofactor in over 300 enzyme systems and serves as a substrate for phosphate transfer reactions in all cells. Several studies show that magnesium metabolism may be altered during and after long-duration spaceflight [20, 35, 72]. After crew members have spent 4–6 months in space, their urinary magnesium is about 45% less than it was before flight [20]. The causes and implications of this are also being evaluated in ongoing ground-based and flight studies.

3.3.1.3. *Hematology and Iron.* Decreased red blood cell (RBC) mass is a consistent finding after short- and long-term flights [73–77]. This "spaceflight anemia" was observed as early as Gemini missions in the 1960s [78]. The initial decrease in RBC mass occurs at a rate slightly greater than 1% per day, with an eventual loss of 10–15% within 10–14 days of flight [73–75]. During the first several days of spaceflight, hematocrit is either unchanged [79] or slightly elevated [73–75]. When elevation is noted, it is not as great as would be predicted from the decrease in plasma volume [17].

A confounding factor in the early flights (before Skylab) was the increased partial pressure of oxygen in the spacecraft cabin [77]. The possibility that hyperoxia-induced peroxidation of RBC membranes was considered, but was ruled out when changes in erythropoiesis were also observed during Skylab [76, 80] and Shuttle missions [74, 75], in which the partial pressure of oxygen in the cabin was similar to that of the Earth's atmosphere [14, 76].

An early hypothesis for the cause of decreased RBC mass was that RBC synthesis in space was understimulated compared to synthesis on the ground [77]. Decreased release of mature RBCs into the circulation is associated with a decrease in circulating erythropoietin concentrations. Serum erythropoietin decreased in the first few days of spaceflight, but it returned to preflight levels later and iron turnover is unchanged during flight [74, 75], indicating that synthesis of RBCs and hemoglobin is unchanged.

Nevertheless, the release of new RBCs is halted upon entry into weightlessness [74, 75, 81], and newly released RBCs are selectively removed from the circulation [81]. These nascent cells are larger than the more mature circulating RBCs, allowing their selective destruction [81]. Removal of mature RBCs from the circulation is unchanged during flight [75].

Indices of iron metabolism and erythropoiesis return toward normal relatively quickly (days) after landing, although the replenishment of RBC mass

may take several weeks. A dilutional "anemia" often occurs after flight [79], with the disproportionate return of plasma volume before the repletion of RBCs. For example, a 3–5% decrease in hematocrit between landing (R + 0) and R + 3 days is common after both short- and long-duration flight [79].

Although the in-flight decrease in RBC mass is significant, the efficient postflight recovery suggests that it represents an adaptation to weightlessness. In-flight changes in RBC mass and body fluid volumes reach a new plateau after the first weeks of flight, as shown by data from long-duration flights [14, 82, 83]. The triggering mechanism for these changes is unknown. One hypothesis is that the body senses a decreased requirement for blood volume and adapts accordingly. This may be related to changes in fluid (circulatory) dynamics and reduced gravitational strain on the circulatory system during flight, which may result in easier delivery of oxygen to tissues, or to the decreased plasma volume and increased concentration of RBCs in the first few days of spaceflight. The decrease in RBC mass has no documented functional consequences.

Bed rest studies have not proven suitable models for the hematological changes of spaceflight. Although RBC mass decreases during bed rest, erythropoietin is unchanged and hematocrit increases [84], suggesting that the mechanisms that bring about hematological changes during bed rest are different from those that act during flight. If the reduced RBC mass during flight is caused by the reduced gravitational load on the circulatory system, it is reasonable to assume that bed rest alone would not alleviate these forces, but would only reposition them. In studies involving changes in altitude, however, the descent from high to low altitude induces changes similar to those observed for spaceflight (decreased RBC mass, increased iron storage) [85].

One consequence of the decreased RBC mass is that the iron released when new RBCs are destroyed is processed for storage. This interpretation is based on findings of increased serum ferritin concentrations during and after both short- and long-duration flights. Serum iron concentrations are normal to elevated during and after flight [74, 75]. The implications of excess stored iron during extended-duration spaceflights are currently unknown. Current space food systems provide excessive amounts of dietary iron (over 20 mg/day, Table 1), which have the potential to cause deleterious effects during extended-duration space missions.

The evidence for increased iron storage and excess iron intake during flight pose pathological risks due to the possibility for iron-overload-related issues. Iron-related radicals could form during iron-overload situations, and this could confound damage induced by ionizing radiation and inflammatory-immune injury [86]. Free radical involvement subsequent to elevations in iron stores has also been linked to cardiovascular disease and cancer. Although

the evidence supporting this thesis is contradictory [87, 88], a correlation between coronary heart disease and iron status has been described in a number of recent studies [89–91] and an association between increased incidence of myocardial infarction and increased iron stores (as measured by serum ferritin) has been observed [91, 92]. Increased risk of all cancer types combined and colorectal cancer in particular was associated with high iron stores in a prospective Finnish study [93]. The relationship between iron, lipids, and cancer has also been documented in the Framingham study [94]. A relationship has also been indicated between excessive iron stores and ascorbic acid deficiency; when reductions in ascorbic acid occur, vitamin A and selenium tend to exacerbate iron-induced peroxidation processes [95]. These data suggest that the alterations in erythropoiesis and iron metabolism that occur in microgravity could cause significant changes affecting crew health.

3.3.1.4. *Trace Elements.* The release of zinc from bones (due to demineralization) has been noted in bed rest studies [96, 97]. Similarly, increases in urinary zinc have been noted with increased muscle catabolism in cases of starvation or trauma [98]. The importance of this phenomenon for spaceflight has not been evaluated (nor has the release of other heavy metals from bone during flight, although this has been modeled and proposed as a concern [99]).

The role of copper in maintaining normal immune function seems to be altered during spaceflight [100, 101], despite the fact that serum copper concentrations are unchanged after ISS flights [20]. Additionally, the documented changes in bone status during spaceflight may be exacerbated by copper deficiencies. Anemia of spaceflight is manifested as a reduction in circulating RBC mass with elevations in serum ferritin and iron concentrations [14, 75]. Since copper is required for iron mobilization and absorption, alterations in copper status may affect iron and RBCs during flight.

Selenium has many biological functions, including serving as a cofactor for glutathione peroxidase. Although long-duration spaceflight data have shown that serum selenium is about 10% lower after flight than before launch [20], the serum activity of glutathione peroxidase does not change and the decrease in selenium may not have physiological effects.

Providing adequate amounts of dietary iodine is not a critical issue for spaceflight, but possible effects of the iodine used as a bactericide in the water systems on orbit have been discussed [102].

3.3.2. *Current Research and Plans*

The nutritional assessment protocol on the ISS has been implemented as a medical requirement before and after flight. Because reliance on postflight assessment does not permit evaluation of the time course of changes during

the mission, an experiment protocol was developed and initiated in 2006 to begin collection of blood and urine samples during flight. Blood (and urine) collection during flight is not new, and in fact was first performed on Skylab, where complete metabolic balance studies were conducted. On the ISS, however, despite a continued human presence on the station since 2000, blood-processing hardware has not been available. Specifically, a centrifuge and freezer were not available until 2006. As of this writing, three US crew members have completed on-orbit blood sample collection and processing operations, including phlebotomy (Fig. 4) and blood processing (Fig. 4). In earlier space programs (Skylab, Shuttle, Mir), $-20\,^{\circ}C$ freezers were available to store biological samples, but for the ISS a $-80\,^{\circ}C$ freezer was developed and flown by the European Space Agency (Fig. 4). This equipment marks the beginning of a new era of ISS research, and will be critical for the further characterization of the human response to microgravity as well as countermeasure evaluation and validation.

4. Spaceflight Effects on Physiological Systems

4.1. Bone

4.1.1. *Spaceflight*

Bone loss is a significant concern for space travelers [8, 103–106], partly because it is related to an increased risk of renal stone formation [70, 107–109], which results in large part from release and excretion of bone minerals. Spaceflight-induced bone loss occurs primarily in weight-bearing bone, and the rate of loss is estimated to range from about 0.5% to 1.5% per month [103, 110, 111]. Femoral trabecular bone density is lost at a rate of about 2.5% per month, which is faster than the rate of cortical bone loss. The higher percentage loss of trabecular bone has consistently been found in spaceflight [110, 112], animal models of microgravity (hindlimb suspension) [113], and spinal cord injury [114]. The amount of loss varies considerably within subjects (at different bone sites) and between subjects. Data from 14 ISS crew members (mission duration was 4.3–6.5 months) show that bone was lost from the lumbar spine and hip at a rate of 0.8–0.9% per month and 1.2–1.5% per month, respectively [110]. After long-duration flights, the cumulative loss of bone tissue is about 2.9% in calcaneus and is greatest in trochanter (about 7–9%) [111, 115]. Changes in bone architecture occur with remodeling after landing and are worthy of concern, but are not yet well defined or understood [116]. The rate of postflight recovery of calcium and bone mineral density is much slower than the rate of loss; recovery of lost mineral seems to take two to three times the flight duration [19, 53].

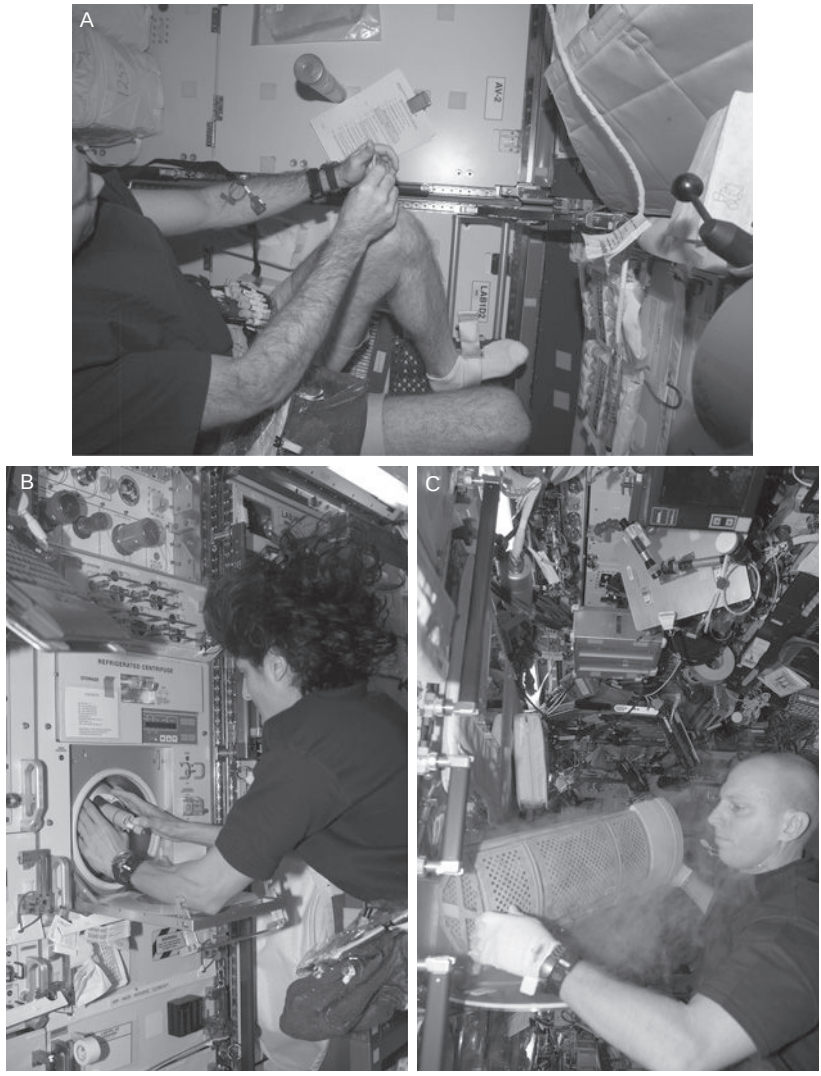

FIG. 4. Collection, processing, and storage of body fluid samples on board the International Space Station. (A) Astronaut Mike Lopez-Alegria, the Expedition 14 commander, collecting a blood sample. (B) Astronaut Sunita Williams, an Expedition 14 flight engineer, processing blood samples with the centrifuge. (C). Astronaut Clayton Anderson, an Expedition 15 flight engineer, storing blood and urine samples in the ''Minus Eighty Laboratory Freezer for ISS'' (MELFI).

4.1.2. *Biochemistry*

Beyond densitometric evaluations, studies of calcium and other markers of bone metabolism provide evidence of early changes in bone metabolism during spaceflight, long before changes in bone mineral can be detected. The initiating mechanism of bone loss during spaceflight is not completely understood, but a number of factors are likely to contribute. Decreased absorption of calcium from the intestine was observed in Mir astronauts and cosmonauts [19, 53, 68], and may have been related to the decreased concentration of circulating 1,25-dihydroxyvitamin D that was also observed in these crew members [18–20, 53, 68].

Although vitamin D status is a significant concern for space travelers because their diet lacks adequate vitamin D content and skin lacks ultraviolet B light exposure, this is not believed to be related to bone loss. Studies on the Mir space station clearly documented decreased serum 1,25-dihydroxyvitamin D and PTH long before vitamin D stores (as reflected by 25-hydroxyvitamin D) were affected [26, 53]. Supplementation with 400–650 IU vitamin D has not proven effective [20, 26], and studies are underway to determine if greater amounts can maintain vitamin D stores. Nonetheless, maintaining vitamin D stores, while important, will not mitigate the drop in both PTH and 1,25-dihydroxyvitamin D concentrations [26].

Early studies in animal models (primarily young, growing rodents) documented reduced bone formation during flight, with little or no change in resorption. In the 1990s, the collagen cross-links were identified as specific markers of bone resorption and bone alkaline phosphatase as a marker of bone formation. Many studies have subsequently documented increased bone resorption during spaceflight, with excretion of collagen cross-links typically 100–150% above preflight levels [19, 55, 68, 117–119].

Bone formation either remains unchanged or decreases during spaceflight [19, 20]. Increased resorption and decreased or unchanged bone formation, coupled with decreased calcium absorption and increased calcium excretion, yield an overall negative calcium balance and bone mineral loss during long-duration spaceflights [110–112].

4.1.3. *Ground Analogs*

Spaceflight research opportunities are very limited, and in most cases the number of subjects is very small. To supplement data from astronauts, researchers have studied bone loss in several ground-based analogs (models) of spaceflight [2, 103], one of the more common analogs with human subjects being bed rest. The qualitative effects of bed rest on bone and calcium homeostasis are similar to the effects of spaceflight, but the quantitative effects are generally less than (about half) those of spaceflight. The effects

of bed rest on bone include loss of bone mass [120–125], a decrease in calcium absorption [126], an increase in calcium excretion [120, 121, 123, 126–135], an increase in risk of renal stone formation [109, 130, 136, 137], and decrease in the serum concentrations of PTH [120, 127, 128, 132] and 1,25-dihydroxyvitamin D [123, 126–128, 132, 138].

Bone resorption increases during bed rest, as measured by histomorphometry [139] or biochemical markers such as hydroxyproline [103, 126, 133] or collagen cross-links [117, 120, 123, 124, 126, 127, 129, 132, 135, 140–146]. Although the magnitude of changes seen in bed rest studies (~50% increase) is less than that of changes seen during spaceflight (~100–150% increase) after similar amounts of time, the qualitative similarities are striking [120, 132, 141, 147].

Bone formation, as assessed by biopsy and histomorphometry, decreases during bed rest [128, 139]. However, assessment of bone formation by measurement of biochemical markers indicates that bone formation either decreases [142] or remains unchanged [120, 123, 124, 126, 127, 129, 132]. These results likely reflect a difference between site-specific (biopsy) and systemic (biochemical markers) indices of bone formation. After subjects return to typical ambulation following bed rest, their markers of bone resorption return to prebed rest levels, and formation markers generally increase [124, 126, 132].

Bone loss and altered calcium homeostasis also occur in paralyzed individuals [148, 149], and have marked similarities to spaceflight findings. PTH and 1,25-dihydroxyvitamin D concentrations are decreased in patients immobilized secondary to spinal cord trauma [150–153]. These changes probably lead to the decreased intestinal calcium absorption and increased fecal calcium excretion [151] of these patients. Bone resorption markers (collagen cross-links, urinary calcium, and hydroxyproline) are also elevated [151, 154–156], with no change in formation markers [154]. The loss of bone after spinal cord injury seems to stabilize after about 25 weeks [157].

4.1.4. *Countermeasures*

Calcium mobilization occurring in bed rest studies conducted in the 1940s [136] led to early expectations that bone loss would occur during weightlessness. From almost that point on, investigators have been searching for a means to counteract this loss [104, 105, 158–164], but a flight-validated countermeasure to bone loss has not yet been documented.

Exercise is a common approach to counter both muscle and bone loss in flight [165, 166], although for bone the difficulty seems to be in attaining the force required to stimulate bone to a degree at which loss can be mitigated. Many types of exercises and devices have been studied, alone or in rare cases in combination, with mixed results. Although many ground-based studies

have demonstrated positive effects of exercise (e.g., treadmill, flywheel, weight stacks) on bone (assessed by various means from densitometry to biochemistry) [120, 123, 132, 144, 167–170], flight validation of the same effect has not been possible to date [110]. No doubt many issues contribute to this lack of on-orbit success, including the quantitative difference between bone loss during bed rest and spaceflight and the function, availability, and utilization of on-orbit hardware. The question of whether the same degree of exercise effectiveness can be reached during flight as in ground analogs is yet to be answered.

Pharmacological agents, the most common being the bisphosphonates, have also been tested for their ability to mitigate weightlessness-induced bone loss. Many ground analog studies (including bed rest studies and studies of patients immobilized because of spinal cord injury or other reasons) have been conducted, with generally positive findings [123, 141, 144, 169, 171–174]. However, ongoing discussion and debate surround the relative safety of these compounds for use in otherwise healthy individuals (astronauts), as opposed to the target population for whom the drugs were developed (patients with disorders such as osteoporosis). In addition to resolving safety concerns, investigators have yet to determine the optimal drug, dose, and schedule of administration during spaceflight. As noted above with exercise, given that the bone loss of bed rest is about half that of spaceflight, there is little reason to believe that the same dose of drug will have the same effectiveness in flight.

Vibration has also received much attention recently in the hope that it can provide a viable musculoskeletal countermeasure [175–177], and the initial ground-based evaluations are underway. One related study has shown that vibration will counteract hypercalciuria induced by excessive dietary protein [178] (see Section 4.1.5). As with all proposed countermeasures, vibration must first be proven effective in ground analog studies (such as bed rest), and if clearly successful, then in-flight validation studies can be conducted.

Under the assumption that lack of gravity is the stimulating factor in the bone loss of spaceflight, replacement of gravity by centrifugation (artificial gravity) has been proposed as a multisystem countermeasure [179], particularly for bone. Some of the artificial gravity studies have relied on short-radius centrifuges [180], others on rotating exercise devices [181] intended to provide gravitational impact as well as physical exercise. Artificial gravity or hypergravity has shown to positively affect bone in human and some animal studies [182–184]. Vernikos *et al.* reported that intermittent exposure to 1 G_z (by standing or walking) during a 4-day head-down-tilt bed rest was effective in preventing elevated urinary calcium that typically occurs during bed rest [185]. The optimal artificial gravity prescription for bone, including dose, duration, and frequency of centrifugation, remains to be clarified.

4.1.5. *Dietary Influences*

Bone health is most commonly associated with calcium and vitamin D status, and both of these are significant nutritional concerns for spaceflight [186, 187]. Unfortunately, while deficiency of these nutrients will induce (or in spaceflight, exacerbate) bone loss, providing them in excess is not considered a viable countermeasure against bone loss. Vitamin D is a good example of this—whereas vitamin D deficiency will lead to mobilization of bone calcium, excess will not stop bone loss. Beyond calcium and vitamin D, many other nutrients also have an impact on bone health. Whether these nutrients have a role to play in the bone loss of spaceflight is yet to be fully defined. We report here some of the preliminary data, and their potential implications for astronauts and human health in general.

Several dietary factors seem to have an influence on bone via alterations in acid/base balance. Specifically, conditions that induce metabolic acidosis are also commonly associated with altered bone metabolism [188, 189]. Because bone is a substantial reservoir of ions that can buffer excess acid loads, chronic small perturbations of acid/base balance in the body can induce prominent changes in the chemical makeup of bone [190, 191].

Dietary intake can influence endogenous acid production because acid and base precursors (i.e., compounds that yield acid or base after they are absorbed and metabolized) exist in foods [192]. If the diet contains more acid precursors (such as sulfur-containing amino acids [193]) than base precursors, chronic low-grade metabolic acidosis can result [194]. Diets high in protein (and/or sulfur-containing amino acids), particularly ketogenic diets, are commonly associated with increased urinary calcium excretion and lower urinary pH [195, 196] and are also associated with lower bone mineral density [197, 198]. Potassium is the predominant intracellular inorganic cation that balances the charge of organic anions; therefore, dietary potassium intake can be used to estimate the content of base precursors in the diet. Frassetto *et al.* developed a model for estimating net endogenous acid production from the amount of acid and base precursors in the diet [199]. According to their model, renal net acid excretion can be predicted from two dietary components: total protein and potassium. Although some controversy exists about whether high intake of protein is detrimental or beneficial to bone, the resolution to this likely lies in the interactions of protein with other nutrients in the diet such as potassium, calcium, sodium, and other minerals, and with nonnutrients such as phytate and oxalate [191, 200].

In a spaceflight analog, we showed that the ratio of dietary protein to potassium intake was correlated with excretion of both calcium and collagen cross-links [168, 201]. This observation was clear after 2–3 weeks of bed rest, but it was not observed in the same subjects before bed rest. Our hypothesis is

that the impact of protein intake on bone is more pronounced in individuals whose bone is metabolically challenged. That is, in situations likely to cause bone loss, excess dietary protein exacerbates this. In well-fed, generally healthy and ambulatory individuals, this effect is not seen. This may further explain the controversy in the field regarding the impact of protein on bone in otherwise healthy individuals.

Dietary sodium is also known to affect calcium homeostasis [26, 202, 203], and a relationship between sodium intake and renal stone formation is well documented. Sodium intake of stone formers is typically similar to that of controls [204, 205], but high sodium intake has detrimental effects on renal stone risk [206–208]. Increased renal stone risk during and after spaceflight is also well documented [70, 107, 108, 209]. A pharmacological approach to the acid/base effect on bone, specifically provision of potassium citrate, has been tested during flight (Whitson *et al.*, unpublished data) and in ground-based models [137].

One hypothesis about the mechanism of dietary sodium's effect on calcium metabolism is that increased renal calcium excretion is secondary to solvent drag and electrochemical gradients in the kidney [210]. However, many studies document that high dietary sodium chloride leads to increased bone resorption [206, 211, 212], and conversely, that restriction of dietary sodium will reduce bone resorption [213], indicating that sodium affects bone metabolism. The mechanism for increased bone resorption with high dietary sodium intake seems to be related to increased dietary sodium chloride intake having an effect on acid/base balance, with subsequent loss of calcium [207, 214].

Other nutrients also affect bone health, and in some cases have been proposed as potential countermeasures. Vitamin K, as mentioned earlier, may have the potential to mitigate bone loss during spaceflight [26, 172], and in limited flight studies has been shown to have positive effects on bone biochemistry [56, 172]. Very recent evidence suggests that omega-3 fatty acids, commonly found in fish oils, can increase bone density in humans and rats [215–219]. Although preliminary data support this concept in microgravity analogs, additional work is required before omega-3 fatty acids can be tested as a countermeasure during spaceflight.

4.2. Muscle

4.2.1. *Spaceflight*

Exposure to microgravity reduces muscle mass, volume, and performance, especially in the legs, on both long- and short-duration flights [220–224]. Muscle loss during long-duration exploration missions is a critical concern

because of its possible implications for astronaut performance of extravehicular activity (EVA), landing and egress tasks, and tasks required during emergency situations.

4.2.2. *Biochemistry*

Potassium and nitrogen balances became increasingly negative throughout the Skylab flights, but urinary creatinine excretion did not change [35] despite losses of leg volume [225]. Decreased prostaglandin secretion has also been implicated in the loss of muscle tissue during spaceflight, secondary to decreased muscle mechanical stress [226]. Stable isotope studies have shown that whole-body protein turnover increased during short-term spaceflight. Protein synthesis increased, but protein breakdown increased even more [227].

Most studies of human protein metabolism during spaceflight have focused on protein synthesis (mostly because of technical limitations). The increase in protein synthesis in short-term flight is hypothesized by Stein [222] to be related to physiological stress, as indicated by increased urinary cortisol during flight [17, 226]. These findings are similar to those found in catabolic patients.

On long-duration Mir flights, conversely, investigators have noted decreased rates of protein synthesis [228]. Protein synthesis was, however, directly correlated with energy intake, suggesting that the reduced protein synthesis was related to inadequate energy intake [228].

Tracer turnover studies have suggested that reduced protein synthesis was the main factor in lean tissue loss during spaceflight [221], but these studies have also shown that protein breakdown was greater as well, particularly during periods of increased stress. Muscle proteolysis occurs mainly in the early stages of spaceflight, and may level off as the duration increases [221, 227]. Profound muscle breakdown can be caused by elevated cortisol in conjunction with decreased testosterone, as seen in trauma patients [221] and during spaceflight [17, 229]. Cortisol treatment alone causes changes in protein indistinguishable from those of fasting [221]. In rats flown on the Space Shuttle, as well as those undergoing hindlimb suspension, evidence exists that activation of the ubiquitin–proteasome pathway was increased [230]. Multiple components of the ubiquitin-proteasome system are up-regulated during spaceflight [230, 231], suggesting that spaceflight-induced muscle proteolysis may be associated with activation of the ubiquitin–proteasome system. Thus, weightlessness-induced muscle loss may be mechanistically similar to other muscle-wasting conditions, including those caused by cancer and sepsis. These findings, along with the commonly noted deficit in energy intake in spaceflight and increased levels of cytokines and other markers of metabolic stress [222], suggest that proteolysis plays a role in lean tissue loss during spaceflight.

Evaluation of plasma and urinary amino acids suggests that they do not provide a clear indication of muscle metabolism. An increase in plasma amino acids was noted in cosmonauts after flights of 2–63 days [232], and limited Shuttle (short-duration) flight data indicate a tendency for plasma concentrations of branched-chain amino acids to be greater during flight than before flight [233]. Data from short-duration flights also showed that little or no change occurred in urinary amino acid profiles [25]. Skylab studies, on the other hand, did reveal increases in excretion of amino acid metabolites [234], suggesting that contractile proteins of skeletal muscle were degraded in weightlessness.

4.2.3. *Ground Analogs*

Disuse atrophy of muscle in space is likely related to changes in whole-body protein turnover. Ground-based studies have shown that whole-body protein synthesis decreases about 10% during short-duration (2 weeks) bed rest [235, 236], and half of that decrease could be accounted for by the leg muscles [236]. Excretion of 4-pyridoxic acid, a vitamin B_6 metabolite, increased during bed rest [237], suggesting that metabolically active muscle tissue was lost.

While the majority of ground-based studies have identified decreased protein synthesis as the likely cause of muscle loss, flight studies generally point to increased proteolysis. These differences may relate to a number of variables. Dietary intake is one major difference between the two types of studies. Ground-based studies typically have prescribed and controlled dietary intakes or are designed to maintain body mass, whereas space crews often do not consume adequate energy. Another difference is the potential variability in stress levels from this type of study, both flight and ground-based. An increase in stress hormones (such as cortisol) is typically, but not always, associated with spaceflight. Ground-based studies have the potential for increased stress; however, this is not an entirely consistent finding. Experimental approaches to mimic increased stress during bed rest have included administration of exogenous thyroid hormone or cortisol as a means to increase muscle catabolism [238–240].

4.2.4. *Countermeasures – Exercise*

When contemplating muscle loss, the most obvious countermeasure is exercise. The exercise protocols used to date have not succeeded in maintaining muscle mass or strength, or bone mass, during spaceflight. This may, in part, be related to time available for exercise, and/or hardware availability or restrictions (such as speed limitations on treadmills). On Mir flights, crew members differed significantly with respect to in-flight exercise frequency and intensity (because of such factors as mission requirements and personal habits).

However, losses of leg muscle volume, detected immediately after flight by magnetic resonance imaging, were almost 20% in all subjects [241]. Similar findings (wide variations in exercise, lack of difference in bone loss) have also been documented for bone and calcium loss [19].

Resistive exercise protocols have been proposed to aid in the maintenance of both muscle and bone during flight. Success with these protocols in flight analog studies [120, 167] has yet to be repeated in flight, in part because of limitations in available exercise hardware [242, 243]. Exogenous testosterone administration during bed rest studies has maintained muscle mass and protein balance, but with no effect on muscle strength [244].

The optimal in-flight exercise prescription (time and type) needs to be developed on orbit. Given the constraints on time and hardware, this prescription may ultimately require a combination of exercise and other countermeasures (such as dietary supplementation and pharmacological agents).

4.2.5. *Countermeasures – Dietary Influence*

In early studies, researchers sought to determine if additional dietary protein could mitigate the muscle loss of weightlessness, but it did not [245]. A number of amino acid (and other nutrient) mixtures have also been researched, with generally positive effects on protein synthesis, muscle loss, and muscle strength [239, 240, 246–250]. One side effect noted in these studies, however, is that the amino acid load seems to have a negative impact on bone metabolism [251].

Administration of omega-3 fatty acids, specifically eicosapentaenoic acid (EPA), attenuates muscle loss associated with hypercatabolic states, including starvation and cancer cachexia. Mechanistic studies indicate that EPA prevents muscle protein catabolism by downregulating proteolysis regulated by the ubiquitin–proteasome system [252]. Even before the mechanisms had been fully defined, EPA entered clinical trials in the United Kingdom for patients with pancreatic cancer and profound cachexia. The results were striking, with either pure EPA [253] or fish oil capsules [254] attenuating loss of lean body mass. No other therapy has ever achieved this, and this work has stimulated much clinical interest throughout both Europe and the United States. Given the abundance of data showing that EPA can successfully prevent muscle atrophy during other muscle-wasting conditions such as cancer or sepsis, there is a high likelihood that it can do this during spaceflight.

4.3. Fluid Balance

Fluid and electrolyte homeostasis is altered during spaceflight [28, 255–258]. The original hypothesis for the mechanism of this effect was that upon entering weightlessness, the human body would experience a headward shift

of fluids, with subsequent diuresis and dehydration. Data from spaceflight experiments have not supported this hypothesis [17, 259–262].

Within hours of the onset of weightlessness (the earliest available data point), a reduction in both plasma volume and extracellular fluid volume occurred [17]. Initially, the decrement in plasma volume (about 17%) was larger than the decrement in extracellular fluid volume (about 10%), suggesting that interstitial fluid volume (the other four-fifths of extracellular fluid besides plasma volume) is conserved proportionally more than plasma volume [17]. The idea that interstitial fluid volume is conserved is supported by rapid decreases in total circulating protein, specifically albumin [17]. This shift of protein, and associated oncotic pressure, from the intra- to the extravascular space would also facilitate the initial changes in plasma volume [17].

After the initial adaptation, extracellular fluid volume decreased between the first days of flight and the 8th to 12th days of flight, from the initial 10% below preflight levels to about 15% below preflight levels [17]. Plasma volume was partially restored during this period, from the initial 17% below preflight levels to about 11% below preflight levels [17], and it has been found to remain 10% to 15% below preflight levels even for extended-duration flights [76].

It is hypothesized that the extravascular shift of protein and fluid represents an adaptation to weightlessness, and that after several days, some of the extravascular albumin has been metabolized, with a loss of oncotic force and a resulting decrease in extracellular fluid volume and increased plasma volume [17]. This loss of extracellular protein (either intra- or extravascular), and associated decreased oncotic potential, probably plays a role in postflight orthostatic intolerance, which has been considered to result partly from reduced plasma volume at landing [263]. Furthermore, the loss of protein may explain why fluid loading alone does not restore circulatory volume [264], as no additional solute load exists to maintain the fluid volume.

The effect of spaceflight on total body water has been evaluated to assess hydration. The total body water of Shuttle and Skylab astronauts decreased about 1% during flight [17, 265], and the percent of body mass represented by water did not change. Thus, the often-hypothesized weightlessness-induced diuresis and subsequent dehydration do not exist [17, 255–257, 262, 266–269], for a number of possible reasons. Operational constraints have made it difficult to document urine volume accurately on the first day of spaceflight. However, on Space Shuttle missions, urine volume on the first three days of flight was significantly less than preflight volume, and tended to be less than preflight volume throughout the flight [17]. Urine volume on a weeklong flight to Mir was also less than preflight volume [270]. During the first week of the 59- and 84-day Skylab flights [35], urine volume was less than it was before flight, and for the remainder of the mission it was unchanged from

preflight levels. Decreased fluid intake likely accounts for the decreased urine volume, which was accompanied by little or no change in total body water.

As mentioned above, the percent of body mass represented by total body water is relatively unchanged during flight [17]. However, on a volume basis, the change (decrease) in extracellular fluid volume was found to be greater than the change (or lack of change) in total body water [17]. Thus, by difference, intracellular fluid volume increased during spaceflight. This had been previously hypothesized from ground-based studies [271] and observed postflight for Apollo crew members [269]. The mechanism for a spaceflight-induced increase in intracellular fluid volume is unknown. One possible explanation is that a shift in fuel utilization results in increased glycogen storage, a condition known to increase cellular water content.

4.4. Endocrine and Immune Function

The interrelationships of nutrition with bone, muscle, and fluid-regulating systems seem to be less complex than its interrelationships with the endocrine and immune systems. A full characterization of the endocrine response to spaceflight has not been possible to date, albeit not for lack of trying. Small numbers of subjects, individual and mission variability, and many confounding factors impede a clear understanding. A larger challenge seems to be the fact that endocrine responses to actual spaceflight are different from responses to ground-based analogs, and this presents another confounding factor when trying to evaluate the available literature.

Bed rest is known to blunt insulin responsiveness [272–274]. Some of the small amount of data available indicates that spaceflight has a similar effect [275], and some does not [226, 276]. Testosterone levels are reduced during flight [277, 278], and a similar, though transient, effect was observed in bed rest [279]. The secondary effects of these endocrine changes on metabolism of fuel, maintenance of muscle and other systems, and other processes are unknown.

Nutrition and immune system function have been clearly linked in Earth-based research. Changes in immune function associated with spaceflight and spaceflight analogs have also been reported [280], but the interrelationships with nutrition have yet to be evaluated beyond speculation in review articles such as this one [281–283]. Although the role of inadequate nutrition in most physiological systems during spaceflight has not been systematically studied, the paucity of knowledge about nutrition and the immune system is especially profound. Further intertwined in the immune/nutrition axis are stress hormone effects [250, 284–286], which not only confound this relationship but also have a clear role in spaceflight effects on muscle, bone, and other systems.

5. Environmental Issues

5.1. Radiation

Spaceflight predictably increases radiation exposure of astronauts due to the radiation environment in low Earth orbit. The types of radiation include three main categories: trapped particles of the Van Allen belts, galactic cosmic rays, and solar particles. Some activities, such as EVA, increase an astronaut's exposure to radiation because the EVA suit provides less protection from radiation than the spacecraft does. Radiobiological effects from each component of space radiation have been studied in ground analogs, but the effects of space radiation as a whole are difficult to test in a ground analog because it is a mixed radiation field and the dose rates are low. Modeling has been used to assess risk, but it is difficult for models to take into account the whole-body biological effects and cellular repair responses after radiation exposure that occur after long-duration spaceflight. For instance, a number of studies show that astronauts have elevated levels of markers of oxidative damage after spaceflight, but predicting the precise cause of the damage is complicated. Plasma malondialdehyde, 8-iso-prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and urinary 8-hydroxy-2′-deoxyguanosine (8OHdG) have been measured during and after flight as indicators of lipid peroxidation (malondialdehyde and PGF_2) and DNA damage (8OHdG) [18, 58]. Several investigations show a significant elevation of urinary 8OHdG after long-duration missions [18, 20] but not after short-duration missions of 17 days [58]. Urinary PGF_2 is significantly decreased during flight but elevated about 2.5-fold after flight [58]. Plasma malondialdehyde is increased both during and after flight [58]. Damage to cellular components such as DNA is a complex process and includes direct damage from high-energy particle impacts on the molecules themselves as well as indirect damage from the production of reactive oxygen species [287, 288]. In addition to these markers of oxidative damage, it is also evident that astronauts have an elevated incidence of cataracts, which are typically induced by reactive oxygen species [44]. Apparent increases in oxidative damage observed during and after flight could be caused by a number of factors, including altered repair mechanisms, impaired antioxidant defense systems, and increased oxidative stress.

Ground-based studies show that antioxidants can protect against many types of radiation-induced cellular damage [289–291]. Further studies are required to determine an optimal antioxidant countermeasure to maximize effectiveness.

5.2. Extravehicular Activity: Space Walks

EVA is one of the more enthralling aspects of a spaceflight, and represents a challenge to many systems, as the space suit literally becomes a personal spacecraft. All life support, including oxygen provision, carbon dioxide

removal, temperature control, hydration, and waste management, is provided by the suit. A tear in the suit material could be catastrophic. The low pressure of the suit (4.3 psi) makes a handgrip strenuous exercise. Food provision is extremely limited or impossible, and only water is provided to maintain hydration. The thickness of the suit becomes the only protection against the temperature extremes of space (which depend on the phase of orbit, light, or dark), and it provides a thin layer of protection against radiation exposure.

In addition to the increased radiation exposure during EVAs, the environment inside the suit can promote oxidative damage. Because of the low suit pressure, protocols have been developed to minimize or eliminate the potential for decompression sickness [292]. A "prebreathe protocol" typically includes a 2.5-hour period of breathing >95–100% oxygen to reduce this risk. After the 2.5-hour prebreathe, astronauts are typically exposed to hypobaric 100% oxygen for 6–8 hours during EVA. Similarly, during training for EVAs, crew members breathe a hyperoxic gas mixture (Nitrox) composed of 40% oxygen and 60% nitrogen. They breathe this under hyperbaric conditions of 1.5–2.0 atm (about 20–30 psi) of pressure (at a depth of 15–35 ft). Dives for EVA training last 6–10 hours, with augmented PO_2 producing near-saturation-type dive conditions. Studies from saturation dives show that oxidative damage is evident under these conditions [293].

Oxidative damage has been linked to cataract risk [294] and other health concerns [295–297] [298–302], including muscle wasting [298] and muscle fatigue [299–302]. Muscle fatigue, particularly in the hands, is an important concern for spacewalking astronauts. Future exploration missions on the Moon and Mars are a top priority for NASA, and current designs are going to have astronauts perform 6- to 8-hour EVAs several times per week. Since EVA crew members will be exposed to several types of oxidative damage, developing an effective countermeasure to mitigate the oxidative damage effects will be critical to astronaut health.

6. Future Exploration Missions

After 45 years of human spaceflight and a great deal of space life sciences research, much has been learned about human adaptation to microgravity exposure. From a nutrition perspective, critical questions remain regarding the nutrient requirements for extended-duration missions and the ability of nutrients to serve as countermeasures to mitigate some of the negative effects of spaceflight. Initial studies are underway to better understand nutritional requirements in microgravity, the stability of nutrients in foods stored in space, and oxidative damage and how to counteract it.

For lunar missions, a key question for all physiological systems is whether 1/6-gravity will protect astronauts from the effects of microgravity. The radiation exposure of missions outside low Earth orbit, as well as the desire to conduct extensive lunar exploration involving crew members wearing space suits, also poses critical mission-specific challenges. The design of future lunar space suits is currently underway, and one of the considerations is expanding the ability to provide nutrition during EVA, either by making a nutritional beverage available in the suit or by making it possible for an astronaut to easily exit the suit for a snack or lunch.

Mars missions will require additional technological and biomedical advances. Current scenarios, based on existing propulsion technology, are for missions of about 2.5–3 years, with 6 months of transit to Mars and another 6-month voyage to return. On these flights, early return will not be possible, and thus *in situ* medical capabilities are needed. Determining what diagnostic testing is required and developing technologies to allow such testing in microgravity or 1/3-gravity will be challenging, to say the least. From a food perspective, storage of foods for up to 5 years will be required (as much of the food as possible will be sent ahead of the crewed mission), and ensuring adequate nutrient content at the time of consumption will be critical. For astronauts depending for months to years on a closed food system, any nutrient deficiency or excess could be catastrophic. The question of *in situ* production of food is often raised, but this will bring another set of challenges, and risks if the crew depends on crops for a given nutrient or set of nutrients. Crop failure is not an option.

If requisite nutrients are not obtained by the body for any reason, muscle and bone loss will proceed unabated, despite any exercise or pharmacological countermeasures. If vitamin C (or any other vitamin) is not stable under conditions of long-duration storage and the radiation exposure of deep space, modern astronauts could suffer from scurvy (or other diseases) as did early explorers on Earth.

Nutrition is critical for health, on Earth and in space. As we approach the next phases of exploration beyond this planet, we need to fully understand nutritional requirements in these unique environments to ensure optimal health and mission success.

Acknowledgments

The authors thank Jane Krauhs for editorial assistance.

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BIOMARKERS OF LIVER FIBROSIS

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Author disclosure statement: T.P. is the inventor and has a capital interest in Biopredictive, the company marketing FibroTest, ActiTest, SteatoTest, NashTest, and AshTest. M.M. is a Biopredictive employee

0065-2423/08 $35.00
DOI: 10.1016/S0065-2423(08)00404-6

Abbreviations

APRI	aspartate aminotransferase/platelets ratio index
FT	FibroTest™
AT	ActiTest™
ST	SteatoTest(all Biopredictive, Paris, France; in United States: HCV-FibroSure™, HBV-FibroSure™, ALD-FibroSure™, ASH-FibroSure™ LabCorp, Burlington)
FM	FibroMeter
FSP	FibroSpect II
ELF	Enhanced liver fibrosis
HS	HepaScore
FS	FibroScan

1. Abstract

Liver biopsy, due to its limitations and risks, is an imperfect gold standard for assessing the severity of the most frequent chronic liver diseases. This chapter summarized the advantages and the limits of the available biomarkers of liver fibrosis. Among a total of 2237 references, a total of 14 validated biomarkers have been identified between 1991 and 2007. Nine were not patented and five were patented. FibroTest™ (FT) was the most studied test with 33 different populations including 6549 patients and 925 controls. The mean diagnostic value for the diagnosis of advanced fibrosis assessed using standardized area under the receiver operating characteristics (ROC) curves was 0.84 [95% confidence interval (CI), 0.83–0.86], without significant difference between the causes of liver disease, hepatitis C, hepatitis B, alcoholic or nonalcoholic fatty liver disease. High-risk profiles of false negative/ positive of FT are present in 3% of populations, mainly Gilbert syndrome, hemolysis, and acute inflammation. FT has higher accuracy than aspartate aminotransferase/ platelets ratio index (APRI), the most used nonpatented test. No significant difference has been observed between the five patented tests. A quality score has been assessed in order to compare the quality of fibrosis biomarkers.

Neither biomarkers nor biopsy are sufficient alone to take definitive decision in a given patient and all the clinical and biological data must be taken into account. Due to the evidence-based data, health authorities in some countries have already approved validated biomarkers as first-line procedure for the staging of liver fibrosis.

This overview of evidence-based data suggests that biomarkers could be used as an alternative to liver biopsy for the assessment of fibrosis stage in the four more common chronic liver diseases: C virus (HCV), hepatitis B virus (HBV), hepatitis nonalcoholic fatty liver disease (NAFLD), and alcoholic liver disease (ALD).

Neither biomarkers nor biopsy are sufficient alone to take definitive decision in a given patient and all the clinical and biological data must be taken into account.

2. Introduction

The consensus conference statements not only recommended liver biopsy in the management of almost all patients with chronic liver diseases related to hepatitis C, hepatitis B, ALD, and NAFLD, but also underline the necessity of developing reliable noninvasive tests [1]. Numerous studies strongly suggest that due to the limitations [2–8] and risks of biopsy [9], as well as the improvement of the diagnostic accuracy of new noninvasive biomarkers, liver biopsy should no longer be considered mandatory as a first-line estimate of fibrosis in these most frequent chronic liver diseases [10, 11].

Practices are evolving rapidly and in France a nationwide survey recently found that among 546 hepatologists, 81% used noninvasive biomarker (FibroTest^{TM}-ActiTest^{TM}) (FT-AT) and 32% used elastography (FibroScan^{TM}) (FS), with a dramatic decrease in the use of liver biopsy for more than 50% of patients with chronic hepatitis C, and with a subsequent increase in the number of patients treated [12]. Furthermore a recent overview by French health authorities officially approved noninvasive biomarkers FT and FS as first-line estimates of fibrosis in patients with chronic hepatitis C, recommended reimbursement by social security, and approved liver biopsy only as second-line estimate in case of discordance or noninterpretability of noninvasive markers [13]. An updated overview is pending for other chronic liver diseases at the end of 2007 [13].

The aim of this chapter is to summarize the advantages and the limits of the available biomarkers of liver fibrosis.

The present authors belong to the same group and are actively involved in the development of these biomarkers, with a possible conflict of interest. Therefore, we have identified for this overview three categories of studies:

studies including only the present authors (nonindependent studies), studies not including the present coauthors (independent studies), and the studies combining independent authors and the present coauthors (mixed studies).

3. Methods

3.1. Design of the Overview

We updated previous overviews and meta-analyses of biomarkers of advanced liver fibrosis. The same methods were used and detailed elsewhere [14–17]. The main nonpatented and patented biomarkers have been reviewed but the specific aim of the present overview was to focus on the professional patented fibrosis biomarkers.

3.2. Search Strategy

We searched MEDLINE with the key words "liver fibrosis serum marker" with the limit "human" (July 2007). We hand-searched key journals (*Gastroenterology, Hepatology, Journal of Hepatology, Gut, Journal of Viral Hepatitis,* and *American Journal of Gastroenterology*) from February 2001 to July 2007 to validate the search, as well as the abstract books of the American Association and European Association for the Study of Liver Disease annual meetings.

3.3. Inclusion and Exclusion Criteria

To select published studies, we used the Standards for Reporting of Diagnostic Accuracy (STARD) criteria and the Cochrane Database of Systematic Reviews (CDSR) methods [18]. Only studies with at least two original studies for the diagnosis of advanced fibrosis have been preincluded and only patented biomarkers were analyzed in details. Several previous overviews and direct comparisons have demonstrated that panels were superior to any single biomarker for the diagnosis of advanced fibrosis [10–13, 19–20]. Several direct comparisons (including independent studies of the FT inventor) have been published between FT, the most used patented panel, vs simple noninvasive tests and all observed a greater accuracy of FT vs APRI [13, 21–23] and of FT vs Forns index [13, 23–24]. In order to update these previous results, a new meta-analysis of FT vs APRI, which is the most used nonpatented panel, has been performed.

We excluded all studies except those that included patients with chronic liver diseases; stated that all patients had had the biomarker and liver biopsy;

provided data for true positives and negatives, false positives and negatives, and AUROCs for advanced fibrosis; stated that the biomarker had been assessed blind to the biopsy; and stated the method used for defining the degree of fibrosis. We were careful to avoid including data from duplicate publications. Studies published only with an abstract with less than 500 cases were excluded, as their inclusion will probably not change the overview of published studies. We excluded biomarkers combining other nonbiochemical components such as alcohol consumption, but not the age and gender adjustments.

3.4. Data Extraction

To allow comparisons between causes of liver disease in the studies, we categorized them into five classes: patients with CHC, CHB, ALD, NAFLD, and mixed causes.

We extracted, from the published study, whether the study was performed by the biomarker inventor group (yes, no, mixed groups including inventor). Study inclusion was never dependent on the result of the noninvasive test under investigation.

3.5. Statistical Analysis

The main end point was the biomarker value for the diagnosis of advanced fibrosis [bridging fibrosis or stages F2, F3, F4 according to the METAVIR scoring system [25]), as assessed by the area under the receiver operating characteristics curve (AUROC)].

A significance level of 5% was used as the alpha risk. Each estimate was given with its 95% CI. Comparisons of the odds ratio and of percentages between strata were performed using their 95% CI. The primary analysis was per patient. We used a random effects model for the primary meta-analysis to obtain a summary estimate for the AUROCs with a 95% CI of biomarker compared with liver biopsy.

The AUROC was used as a measure of discrimination, estimated using the empirical (nonparametric) method by DeLong *et al.* [26], and was compared using the paired method by Zhou *et al.* [27]. All analyses are performed on NCSS software (Kaysville, Utah).

Meta-analysis was performed twice when details were available, once according to the absolute value of the observed AUROCs (ObAUROCs) and once according to the AUROCs standardized for the spectrum of fibrosis stages (AdAUROC). We previously demonstrated that the AUROCs were highly related to the difference between the mean fibrosis stages in the

advanced fibrosis and nonadvanced fibrosis groups (DANA); the AdAUROC is the AUROC adjusted for the difference of the observed DANA vs a standard DANA of 2.5 fibrosis METAVIR units (DANA = 2.5 if there was a uniform prevalence of 0.20 in each of the five stages); all the AUROCs were adjusted to a DANA of 2.5 using the formula: AdAUROC=ObAUROC+(0.1056) (2.5−ObDANA) [28, 29].

We compared the biomarkers when there were at least two independent direct comparisons in the same disease specific population. This was possible only in patients with HCV, and between FT and APRI and between FT and HepaScore (HS) and FibroMeter (FM).

3.6. Role of the Funding Source

There was no specific financial support for this overview, but two of the authors have a potential conflict of interest: T.P. is a consultant and has a capital interest in Biopredictive, the company marketing FT, and M.M. is a full-time employee of Biopredictive.

Biopredictive had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

3.7. Quality Evaluation of Fibrosis Biomarkers

A specific list of 62 items has been elaborated to assess the level of quality of each biomarker (Table 1). The aim of these items was to check the level of reliability of the published evidence-based data for each biomarker as it is performed for a drug. Five items were related to the biomarker rational. Fourteen items were related to the sample size of validation studies. Six items were related to the preanalytical and analytical recommendations. Twenty one items were related to the benefit-risk evaluation vs liver biopsy. Two items were related to the diagnostic validation vs other end point than biopsy. Two items were related to the independent validation of the biomarker. Four items were related to the association of the specific fibrosis biomarker with other injury-specific biomarker. Eight items were related to official approvals, availability, and conflict of interest and cost.

TABLE 1
DEFINITION OF 62 ITEMS ESTIMATING THE QUALITY OF FIBROSIS BIOMARKERS

Criteria	Definition of criteria
Rational	
Liver specificity	Are the components specific of the liver?
Extracellular matrix specificity	Are the components specific of fibrosis?
Proteomics validation	Proteomics studies validating biomarkers components as associated with fibrosis.
False negative	Is the rational for false positive described?
False positive	Is the rational for false negative described?
Sample size	
Number of studies	
Overall	Number of studies published (prospective/retrospective).
HCV	Specific studies in chronic hepatitis C.
HBV	Specific studies in chronic hepatitis B.
NAFLD	Specific studies in nonalcoholic fatty liver disease.
ALD	Specific studies in alcoholic liver disease.
Other	Other causes of chronic liver disease: hemochromatosis, autoimmune hepatitis, primary biliary cirrhosis. . ..
Mixed	
Number of patients	Several causes of liver disease included.
Overall	Overall number of patients included in published studies.
HCV	Number of patients in chronic hepatitis C.
HBV	Number of patients in chronic hepatitis B.
NAFLD	Number of patients in nonalcoholic fatty liver disease.
ALD	Number of patients in alcoholic liver disease.
Other liver diseases	hemochromatosis, autoimmune hepatitis, primary biliary cirrhosis. . ..
Number of controls	Healthy controls, blood donors, permitting references range.
Preanalytical recommendations	
Fasting	Variability of results according to fasting/nonfasting.
Storage period	Variability according to storage period.
Storage temperature	Variability according to storage temperature.
Analytical recommendations	
Intralaboratory variability	Impact of parameter assay variations.
Intrapatient reproducibility	Intrapatient reproducibility.
Interlaboratory variability	Variability according to laboratory, kits, and analyzers.

(*continues*)

TABLE 1 (*Continued*)

Criteria	Definition of criteria
Diagnostic methods vs biopsy	
Accuracy or AUROCs	Accuracy or area under the ROC curves (AUROCs).
Linear correlation with fibrosis stages	Linear association with validated staging scoring system.
Intermediate stages	Accuracy similar between extreme stages or intermediate stages.
Adjacent stages	Accuracy between adjacent stages evaluated.
Adjusted AUROCs	Accuracy evaluated according to prevalence of fibrosis defining advanced or nonadvanced fibrosis.
Meta-analysis vs biopsy	Published meta-analysis vs biopsy.
Specificity in normal population	Healthy controls, blood donors.
False positive	Percent of false positive and causes.
False negative	Percent of false negative and causes.
Predictive values	Estimate of predictive values according to cut-offs.
Security algorithms	Procedures for identifying high-risk false positive/negative.
Applicability	Estimates of percentage of patients without high risk of false positive/negative.
Quality biopsy	Details concerning length of biopsy sample and fragmentation.
Analysis of discordance	Analysis of discordant cases between the investigated biomarker and biopsy and other estimates, and of the cause of failure.
Postmarketing studies	Analysis of applicability and discordance in large postmarketing studies.
Paired studies	Analysis of paired samples with baseline and follow-up.
Prognostic studies	Prognostic value of biomarkers vs biopsy staging.
Portal hypertension	Predictive value for portal hypertension.
Difficult to diagnose populations	Specific studies in special populations such as HIV, aged patients, hemophiliac, drug addicts, morbid obese, transplanted, renal failure, and children.
Screening studies	Fibrosis screening studies in high-risk group.
General population	Fibrosis screening studies in general population.
Diagnostic value vs other markers	
Other biomarker	Direct comparisons with other biomarker.
Elastography	Direct comparisons with elastography.
Independent/mixed validation	
Number of studies	Number of studies.
Number of patients	Number of patients.

(*continues*)

TABLE 1 (*Continued*)

Criteria	Definition of criteria
Association with other liver injury biomarkers	
Necroinflammatory activity	Association with a biomarker of viral necrosis and inflammation.
Steatosis	Association with a biomarker of steatosis.
Nonalcoholic steatohepatitis	Association with a biomarker of nonalcoholic steatohepatitis.
Alcoholic steatohepatitis	Association with a biomarker of alcoholic steatohepatitis?
Official approval	
CE Marketed	Self-declaration to European Community.
Guidelines	Recommendation of use by scientific societies.
Health authority	Recommendation of use by health authorities.
Availability	Wordwide availability in hospital and private practice.
Conflict of interest	Participation of coauthors in patent declared in publications.
Cost	Cost known including components and algorithms. (In dollars)
Patent	Patented algorithm.
Reimbursement	Social security or insurance company reimbursement.

4. Results

4.1. Biomarkers Identified

Among a total of 2237 references, a total of 14 validated biomarkers have been identified between 1991 and 2007. Nine were not patented: PGA (Prothrombin, GGT, Apolipoprotein A1) index [30–31], AP index [20, 32], Bonacini index [20, 33], Pohl score [20, 34], Forns index [13, 24, 35], APRI [13, 20, 21, 36], MP3 index [13, 37–39], FIB-4 [13, 40,41], and FibroIndex [42,43]. Five were patented: FT, FibroSpect II (FSP), enhanced liver fibrosis (ELF), FM, and HS. Among these panels the number of components ranged from two to seven (Table 2).

4.2. Evaluation of Patented Biomarkers

4.2.1. *Details of Publications*

A total of 33 different populations have been studied for FT, including 6549 patients and 925 controls (Table 3A); 4 studies for FSP, including 463 patients; 2 studies for ELF, including 1041 patients; 3 for FM, including 1134

TABLE 2
SERUM MARKERS OF HEPATIC FIBROSIS WITH AT LEAST TWO VALIDATIONS

Index [Refs.]	Year of first publication	Key leader	Components	Liver disease
Not patented				
PGA [30–31]	1991	Poynard	PI, GGT, ApoA1	ALD
AP [20, 32]	1997	Poynard	Plt, age	HCV
Bonacini [20, 33]	1997	Lindsay	Plt, ALT, AST,	HCV
Pohl [20, 34]	2001	Pohl	Plt, AST	HCV
Forns [13, 24, 35]	2002	Forns	Plt, cholesterol, age	HCV
APRI [13, 20, 21, 36]	2003	Lok	Plt, AST	HCV
MP3 [13, 37–39]	2004	Leroy	PIIINP, MMP1	HCV
FIB-4 [13, 40–41]	2006	Sterling	Plt, AST, ALT, age	HCV/HIV
FibroIndex [42–43]	2007	Koda	Plt, AST, gamma-globulins	HCV
Patented				
FT/FS [14–17, 44–67]	2001	Poynard	A2M, haptoglobin, ApoA1, Bilirubin, GGT, age, gender	HCV, HBV, ALD, NAFLD, HIV
FSP [49, 68–70]	2004	Oh	A2M, HA,TIMP-1	HCV
ELF [49, 71]	2004	Rosenberg	HA, PIIINP, TIMP-1	Mixed
FM [39, 49, 56]	2005	Cales	Plt, AST, A2M, HA, PI, age, gender	Mixed
HS [39, 56, 72]	2005	Adams	A2M, HA, GGT, age, gender	HCV

APRI, Aspartate aminotransferase/platelets ratio index; GGT, gamma-glutamyltransferase; A2M, alfa2-macroglobulin; ALD, alcoholic liver disease; NAFLD, nonalcoholic fatty liver disease; TIMP, tissue inhibitors of metalloproteinases; MMP-1, matrix metalloproteinases; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HA, hyaluronic acid; ApoA1, apolipoprotein A1; PI, prothrombin index; FSP, FibroSpect II; ELF, enhanced liver fibrosis; HS, HepaScore; FT, FibroTest; FS, Fibrosure; FM, Fibrometer; FIB-4, 4 markers index; AP, age platelet; Plt, platelet; HCV, hepatitis C virus; MP3, metallo protease 3; HBV, hepatitis B Virus.

patients; and 3 for HS, including 757 patients. Only FT had a specific study for each of the four most frequent chronic liver diseases (Table 3B).

4.2.2. *Diagnostic Value and Meta-Analyses*

For FT, a recent meta-analysis included a total of 29 studies pooling 6378 subjects with both FT and biopsy (3501 HCV, 1457 HBV, 267 NAFLD, 429 ALD, and 724 mixed) [14–17] and individual data were analyzed in 3282 patients [17]. The mean of the ObAUROCs was 0.80 (95% CI, 0.78–0.82) and the mean standardized AUROC was 0.84 (95% CI, 0.83–0.86), without

TABLE 3
CHARACTERISTICS OF THE PATENTED BIOMARKERS DIAGNOSTIC STUDIES FOR THE STAGING OF HEPATIC FIBROSIS IN PATIENTS WITH CHRONIC LIVER DISEASE

First author [Refs.]	Year	Number of patients	Methodology	AUROC SE	Independent
A. FibroTest					
HCV					
Imbert-1 [44]	2001	189	Prospective, single center, training cohort	0.84 0.03	No
Imbert-2 [44]	2001	134	Prospective, single center, validation cohort	0.87 0.03	No
Poynard-1 [45]	2001	299	Retrospective, multicenter, randomized trial	0.74 0.03	Mixed
Poynard-2 [46]	2003	352	Retrospective, multicenter, randomized trial, before treatment	0.73 0.03	Mixed
Poynard-3 [46]	2003	352	Retrospective, multicenter, randomized trial, after treatment	0.77 0.03	Mixed
Rossi [47]	2003	125	Prospective, multicenter	0.74 0.05	Yes
Myers-1 [48]	2003	130	Retrospective, single center, HCV-HIV coinfection	0.86 0.04	No
Castera [22]	2005	183	Prospective, single center	0.84 0.03	Yes
Cales-2 [49]	2005	120	Prospective, single center, validation cohort	0.86 0.06	Yes
Coletta [50]	2005	40	Prospective, multicenter, PNALT	Not specified	Yes
Varaut-1 [51]	2005	50	Retrospective, single center, dialysis patients	0.53 0.04	Yes
Varaut-2 [51]	2005	60	Retrospective, single center, kidney recipients	0.71 0.04	Yes
Halfon-1 [52]	2006	504	Prospective, multicenter	0.79 0.02	Yes
Sebastiani-1 [53]	2006	65	Prospective, PNALT	0.71 0.04	Yes
Sebastiani-2 [53]	2006	125	Prospective, EALT	0.81 0.03	Yes
Wilso[a] [54]	2006	115	Retrospective, multicenter, 30% HIV	0.74 0.05	Yes
Sene [55]	2006	138	Prospective, single center cryoglobulin-emia vasculitis	0.83 0.03	No
Halfon-2[a] [56]	2007	158	Prospective, single center	0.79 0.03	Yes
Leroy [39]	2007	180	Prospective, single center	0.84 0.03	Yes
Grigorescu [57]	2007	206	Retrospective, single center	0.78 0.02	Yes
Morali [58]	2007	325	Prospective, multicenter	0.85 0.02	Yes

(*continues*)

TABLE 3 (*Continued*)

First author [Refs.]	Year	Number of patients	Methodology	AUROC SE	Independent
HBV					
Myers-2 [59]		209	Prospective (42) and retrospective (167)	0.78 0.04	No
Poynard-4 [60]	2005	214	Prospective	0.77 0.03	Mixed
Sebastiani-3 [23]	2007	110	Prospective	0.85 0.04	Yes
Poynard-5 [61]	2007	924	Retrospective	0.76 0.02	Mixed
ALD					
Naveau [62]	2005	221	Prospective, one center	0.84 003	Mixed
Thabut [63]	2006	208	Prospective, two centers	0.91 0.02	Mixed
NAFLD					
Ratziu-1 [64]	2006	170	Prospective	0.86 0.03	No
Ratziu-2 [64]	2006	97	Prospective	0.75 0.04	Mixed
Psoriasis mixed					
Callewaert [65]	2004	106	Prospective, HCV and alcohol	0.89 0.04	Yes
Cales-1 [49]	2005	478	Prospective, single center, HCV, HBV, ALD	0.82 0.03	Yes
Coco [66]	2007	164	Prospective, HCV, and HBV	0.89 0.05	Yes
Berends [67]	2007	20	Retrospective, psoriasis	0.83 0.05	Yes
B. FibrosSpect II, enhanced liver fibrosis, FibroMeter, HepaScore					
FibroSpect II					
HCV					
Cales-2[b] [49]	2005	120	Prospective, single center	0.86 0.06	Yes
Christensen [68]	2006	142	Retrospective, single center	0.86 0.05	No
Zaman [69]	2007	108	Prospective, single center	0.83 0.06	No
Snyder [70]	2007	93	Retrospective, single center	0.88 0.07	Yes
ELF					
HCV					
Rosenberg [71]	2004	496	Prospective, multicenter	0.77 0.04	No
Cales-2 [49]	2005	120	Prospective, single center	0.83 0.06	Yes
NAFLD					
Rosenberg [71]	2004	61	Prospective, multicenter	0.87 0.10	No
ALD					
Rosenberg [71]	2004	61	Prospective, multicenter	0.94 0.06	No

(*continues*)

TABLE 3 (*Continued*)

First author [Refs.]	Year	Number of patients	Methodology	AUROC SE	Independent
FibroMeter HCV					
Cales-2 [49]	2005	120	Prospective, single center, validation	0.90 0.03	No
Halfon-2 [56]	2007	356	Prospective, two centers	0.78 0.03	Yes
Leroy [39]	2007	180	Prospective, single center	0.86 0.02	Yes
Mixed (HCV, HBV, ALD)					
Cales1 [49]	2005	478	Prospective, single center, training	0.82 0.03	No
HepaScore HCV					
Adams [72]	2005	117	Single center, training	0.85 0.05	No
Adams [72]	2005	104	Multicenter, validation	0.82 0.05	No
Halfon-2 [56]	2007	356	Prospective, two centers	0.76 0.03	Yes
Leroy [39]	2007	180	Prospective, single center	0.79 0.03	Yes

PNALT, persistently normal transaminases ALT; EALT, elevated transaminases ALT.
[a] Only one center was taken as the other center results were published in Halfon-1.
[b] FibroSpect I have been referenced in the article by Cales.

differences between causes of liver disease: HCV 0.85 (0.82–0.87), HBV 0.80 (0.77–0.84), NAFLD 0.84 (0.76–0.92), ALD 0.86 (0.80–0.92), and mixed 0.85 (0.80–0.93). The AUROC for the diagnosis of the intermediate adjacent stages F2 vs F1 (0.66; 0.63–0.68, $n = 2055$) did not differ from that of the extreme stages F3 vs F4 (0.69; 0.65–0.72, $n = 817$) or F1 vs F0 (0.62; 0.59–0.65, $n = 1788$). Sensitivity analysis integrating the independency of authors, length of biopsy, prospective design, respect of procedures, comorbidities, and duration between biopsy and serum sampling did not found significant differences.

4.2.3. *Comparison Between FT and APRI Diagnostic Values*

In six studies (1630 HCV patients), FT was directly compared with APRI [21, 39, 49, 53, 54, 56], five being independent of FT inventor. Meta-analysis demonstrated a greater ObAUROC for FT (0.83; 95% CI, 0.80–0.85) vs APRI (0.76; 95% CI, 0.73–0.80) with 0.06 (95% CI, 0.03–0.09) mean difference ($P = 0.0005$) without heterogeneity ($Q = 6.4$; $P = 0.27$). This significant difference persisted when the nonindependent study was excluded from analysis: 0.05 (95% CI, 0.01–0.09; $P = 0.003$).

4.2.4. *Comparison Between Patented Biomarkers' Diagnostic Values*

None of the meta-analyses of studies comparing directly reached statistical significance. The number of patients was too small to detect a 5% difference between AUROCs.

Between FT and HS (3 studies in 653 HCV patients) the mean difference was 0.02 (95% CI, 0.03–0.07; $P = 0.30$) and after excluding nonindependent study 0.04 (95%CI, 0.01–0.08; $P = 0.12$).

Between FT and FM (3 studies in 653 HCV patients) the mean difference was −0.005 (95% CI, 0.05–0.04; P=0.81) and after excluding nonindependent study 0.0008 (95% CI, 0.05–0.05; $P = 0.97$).

Between FM and HS (2 independent studies in 536 HCV patients) the mean difference was higher 0.04 (95% CI, 0.01–0.08; $P = 0.13$).

4.2.5. *Quality Items*

For FT, the number of studies ($n = 33$) and the number of patients included ($n = 6549$) were much higher than for all the five other tests (Table 4).

For the qualitative items, responses were missing or unsatisfactory in 2/62 for FT, in 32/62 for FSP, in 36/62 for ELF, in 32/62 for FM, and in 37/62 for HS.

5. Discussion

After this overview what are the responses to the most frequently asked questions concerning biomarkers of fibrosis?

5.1. Are the Coauthors Credible Due to Their Possible Conflict of Interest?

During 15 years the first author has performed laparoscopy and liver biopsies; published extensively on the standardization of liver histology [5, 6, 73]; and used biopsy as main criteria published on fibrosis progression, natural history [74], factors associated with fibrosis progression, [75] and on the impact of treatments [76]. Due to the limitations of liver biopsy [9], including its morbidity and mortality [77], the authors have worked on noninvasive biomarkers in order to replace the liver biopsy as the first-line estimate of liver injury. After trying to develop nonpatented fibrosis biomarkers [20, 30], we do believe that diagnostic biomarkers must follow the same professional development than drugs with highest levels of confidence and official approvals. Our nonpatented PGA index [30] has been prescribed

TABLE 4
CRITERIA FOR ASSESSING THE QUALITY OF FIBROSIS BIOMARKERS

Criteria	FibroTest	FibroSpect II	ELF	FibroMeter	HepaScore
Rational					
Liver specificity	Liver only=haptoglobin, A2M bilirubin, GGT; liver and intestine=ApoA1	Liver only=A2M; not organ specific=hyaluronic acid, TIMP-1	Not organ specific=hyaluronic acid, TIMP-1, PIIINP	Liver only=A2M; not organ specific=platelets, prothrombin, AST, hyaluronic acid, urea	Liver only=A2M, bilirubin, GGT; not organ specific=hyaluronic acid
Extracellular matrix specificity	Yes=A2M; partial=ApoA1, haptoglobin	Yes	Yes	Yes=hyaluronic acid	Yes=hyaluronic acid, A2M
Proteomics validation	Yes=haptoglobin, ApoA1, A2M [100]	No	No	No	No
False negative	Yes=haptoglobin and acute inflammation	Not specified	Not specified	Not specified	Not specified
False positive	Yes=bilirubin, Gilbert syndrome, hemolysis, and extra-hepatic cholestasis; GGT and extra-hepatic cholestasis	Not specified	Not specified	Not specified	Not specified
Sample size					
Number of studies					
Overall	33	4	2	3	3
HCV	21	4	1	2	3
HBV	4	0	0	0	0
NAFLD	2	0	0	0	0

(*continues*)

TABLE 4 (*Continued*)

Criteria	FibroTest	FibroSpect II	ELF	FibroMeter	HepaScore
ALD	2	0	0	0	0
Mixed or other liver disease	4	0	1	1	0
Number of patients					
Overall	6549	463	1041	1134	757
HCV	3628	463	616	993	757
HBV	1457	0	61	46	0
NAFLD	429	0	61	0	0
ALD	267	0	64	95	0
Other liver disease	768	0	239	0	0
Number of controls	925	0	0	0	0
Preanalytical recommendations					
Fasting	Yes [89]	No	No	No	No
Storage period	Yes [88]	No	No	No	No
Storage temperature	Yes [88]	No	No	No	No
Analytical recommendations					
Intralaboratory variability	Yes [81–83 88–92]	No	No	No	No
Intrapatient reproducibility	Yes[88]	No	No	No	No
Interlaboratory variability	Yes [81]	No	No	No	No
Diagnostic methods vs biopsy					
Accuracy or AUROCs	Yes	Yes	Yes	Yes	Yes
Linear correlation with fibrosis stages	Yes [14–17, 28, 29]	No	No	Yes [49]	No
Intermediate stages	Yes [14–17, 28, 29]	No	No	Yes [49]	No
Adjacent stages	Yes [14–17, 28, 29]	No	No	No	No
Adjusted AUROCs	Yes [14–17, 28, 29]	No	No	No	No
Meta-analysis vs biopsy	Yes [14–17, 28, 29]	No	No	No	No

(*continues*)

TABLE 4 (*Continued*)

Criteria	FibroTest	FibroSpect II	ELF	FibroMeter	HepaScore
Specificity in normal population	Yes [14–17, 88]	No	No	No	No
False positive	Yes[14–17, 28, 29, 78, 79]	No	No	No	No
False negative	Yes[14–17, 28, 29, 78, 79]	No	No	No	No
Predictive values	Yes[14–17, 28, 29, 78, 79]	Yes [49, 68–70]	Yes [71]	Yes [49]	Yes [72]
Security algorithms	Yes [78]	No	No	No	No
Applicability	Yes [78]	No	No	No	No
Quality biopsy	Yes [78, 28]	Yes	No	Yes [49]	Yes [72]
Analysis of discordance	Yes [78]	No	No	No	No
Paired studies and longitudinal studies	Yes [45, 46, 60, 61, 87]	No	No	No	No
Prognostic studies	Yes [79]	No	No	No	No
Portal hypertension	Yes [94, 95]	No	No	No	No
Difficult to diagnose populations	Yes [48, 50, 51, 54, 55, 98, 99]	No	No	No	No
Screening studies	Yes [93]	No	No	No	No
General population	No	No	No	No	No
Diagnostic value vs other markers					
Other biomarker	Yes[a][21, 39, 49, 53, 54, 56]	Yes	Yes [49, 71]	Yes [39, 49, 56]	Yes [39, 56]
Elastography	Yes [22]	No	No	No	No
Independent/mixed validation/nonindependent					
Number of studies	18/8/7	3/0/1	1/0/1	2/0/1	2/0/1
Number of patients	3134/2667/748	343/0/120	120/0/921	536/0/598	536/0/221
Association with other liver injury biomarkers					
Necroinflammatory activity	Yes (ActiTest)[b] [14, 15]	No	No	No	No
Steatosis	Yes (SteatoTest) [84]	No	No	No	No

(*continues*)

TABLE 4 (*Continued*)

Criteria	FibroTest	FibroSpect II	ELF	FibroMeter	HepaScore
Nonalcoholic steatohepatitis	Yes (NashTest) [86]	No	No	No	No
Alcoholic steatohepatitis	Yes (AshTest) [85]	No	No	No	No
Official approval					
CE Marketed autodeclaration	Yes	No	Yes	No	No
Guidelines	Yes (French Association Liver Disease) [13]	No	No	No	No
Health authority	Yes (Haute Autorité Santé France) [13]	No	No	No	No
Availability	Yes (Worldwide)	No (United States only)	Yes (Europe)	Not specified	Not specified
Conflict of interest declared	Yes	Yes	No[c]	Yes	No[d]
Cost	Yes (120–350$) [13]	Yes (350$)	Not specified	Yes 120$ [13]	Not specified
Patent	Yes	Yes	Yes	Yes	Yes
Reimbursement social security	Yes (France) [13]	No	No	No	No
Total quality score range: 0–62	60	30	26	30	25

ELF = Enhanced liver fibrosis; A2M = alfa2-macroglobulin; GGT = gamma-glutamyltransferase; ApoA1 = apolipoprotein A1; TIMP-1 = tissue inhibitor of metalloproteinases-1; PIIINP = procollagen IIIN-terminal peptide; AST = aspartate aminotransferase; MMP-1 = matrix metalloproteinase 1.

[a] Versus or combined with APRI and FIB-4.

[b] Includes the cost of ActiTest.

[c] Several coauthors of the article (*Gastroenterology* 2004) are coowner of the Bayer patent or have a capital interest in a start-up.

[d] A recent contract has been declared for the US market.

confidentially in hundreds of patients in 17 years in contrast with the patented FT that has been already prescribed in 220,000 patients in less than 5 years (Biopredictive data on file, Castille, personal communication). The first author has a capital interest in the start-up company marketing FT but the patent belongs to the public organization ''Assistance Publique Hôpitaux de Paris.''

5.2. Is the Perfect Fibrosis Biomarker Possible?

Nonexpert physicians and patients are waiting for an almost perfect test that is a biomarker with less than 10% of false positive/negative results and more than 99% of applicability. This is not possible, even with liver biopsy [11]. A 25 mm not fragmented biopsy is obtained in less than 50% of all large series [78] and the rate of false positive/negative of such a 25 mm not fragmented biopsy is still around 20%, for the diagnosis of advanced fibrosis, in comparison with the true gold standard which is the whole liver [4]. Among the discordances observed between biopsy and biomarker estimates of fibrosis, the cause of failure is frequently due to biopsy failure [78, 79]. Therefore, it is an illusion to wait for an almost perfect biomarker with an adjusted AUROC greater than 90% for the diagnosis of advanced fibrosis. This point must be explained to patients and health authorities.

5.3. Are Patented Biomarkers Better Than Nonpatented Biomarker?

When we compared the diagnostic value of the two most popular fibrosis biomarkers the patented FT and the nonpatented APRI, there were significantly higher AUROCs for FT among six studies comparing directly these biomarkers. The advantage of FT is also to have security algorithms in order to reduce the risk of false positive/negative and to have demonstrated the prognostic values. An advantage of APRI is to be cheap and easy to access. A major disadvantage of APRI is to use upper limits of normal for transaminases, which are not standardized and not reproducible between laboratories [21, 80–83]. The other comparisons between biomarkers were not possible because of the limited number of direct comparisons.

Recently Shaheen *et al.* published an overview of the diagnostic accuracy of FT and FS, and an overview of APRI, for the prediction of hepatitis C-related fibrosis[84, 85]. The major limitations of these systematic reviews included the absence of standardization of the AUROCs according to the prevalence of stages defining advanced or nonadvanced fibrosis, the nonevidence-based statements concerning FT, and the absence of detailed discussion of the impossible perfect biomarker. The methodology used by the authors did not take into account the most important source of heterogeneity between studies, which is the different spectrum of fibrosis stages (spectrum bias) [29].

If a study is overrepresented in fibrosis extreme stages (F0 and F4), the marker sensitivity, specificity, and AUROCs will be automatically higher than a study including only adjacent stages (F2 and F1). Standardization analyses should be performed according to these differences in stages prevalence defining advanced and nonadvanced fibrosis (DANA). The indirect AUROCs comparisons are meaningless without these standardizations [29]. This spectrum bias can also explained why, for a given test [29] or for biopsy [4], the AUROCs of cirrhosis vs non-cirrhosis can vary according to the prevalence of non-cirrhotic stages. The DANA is 2.5 both for cirrhosis AUROCs and advanced fibrosis AUROCs if non-cirrhotic stages' prevalences are similar. If the prevalence of cirrhosis is 40% with 0% F3, the DANA will be 3.25 for diagnosis of cirrhosis and only 3.0 for the diagnosis of advanced fibrosis. Therefore, statements of the authors that accuracy for cirrhosis is better than for advanced fibrosis is meaningless without comparisons between adjacent stages [29]. The authors mentioned a prospective study demonstrating a 5-year prognostic value for APRI but forgot to mention that the FT had a significantly higher prognostic value (survival without complications: AUROC 0.96; 95% CI, 0.93–0.97) than APRI (AUROC 0.82; 95% CI, 0.66–0.91; $P = 0.03$) [79, 86].

5.4. Are There Differences Between Patented Biomarkers?

This overview found no significant difference between the AUROCs for advanced fibrosis among the patented biomarkers. Because of the limited number of patients included in the direct comparisons, a clinically significant difference cannot be excluded, particularly between HS with a possible smaller AUROCs (0.04 difference) vs FT and FM.

From the evidence-based data published, several quality items were missing for several patented biomarkers.

For the applicability of biomarkers and the risk of false positive/negative, more data are needed in larger patient populations and controls for hyaluronic acid, tissue inhibitor of metalloproteinases-1 (TIMP-1), PIIIP, and platelets. For TIMP-1, platelets and PIIP more studies must be performed and published for preanalytical and analytical recommendations. Specific studies in difficult-to-diagnose populations must be performed.

The number of patients studied is small (less than 500 and only in HCV patients) for FSP and also small in non-HCV patients for FM, ELF, and HS.

More data are also needed for the diagnostic values of FSP, FM, ELF, and HS in discriminating early fibrosis stages: F0 and controls vs F1 and between F1 and F2.

Another advantage is that the FT can be combined with other validated biomarkers of liver injuries [15–16]: the AT to estimate the grade of necrosis and inflammation in HBV and HCV [14, 15] as well as the grade of steatosis

using the SteatoTest (ST) [87], the presence of alcoholic using the AshTest [88], and the presence of nonalcoholic steatohepatitis using the NashTest [89].

So far only FT has been studied for the following items: FT has been several times studied in paired biopsies during natural history or repeated during follow-up studies [45, 46, 60, 61, 90]. FT has been studied for pre-analytical and analytical variability [81–83, 91–95], the causes of discordance vs biopsy [78], in screening strategy [96], as a marker of portal hypertension [97–98], and for its long-term prognostic value [79]. Contrarily to the other biomarkers, FT has been also studied in special HCV populations such as patients with vasculitis [55], drug-user [54], aged patients [99], HIV coinfected patients [48], patients with hemophilia [100], children [100–101], patients with normal transaminases [50, 102–103] and with renal insufficiency [51].

One weakness of FT is that several components are viewed as "indirect" markers, such as alfa2-macroglobulin (A2M), ApoA1, and haptoglobin. However besides explanatory pathways, they are now evidence-based data using proteomics demonstrating a direct association between these three proteins and fibrosis [104–105]. The main causes of FT misclassifications are the Gilbert syndrome and presence hemolysis observed in less than 5% and almost always identified with the security algorithms [78].

5.5. Are Fibrosis Biomarkers Effective in All Chronic Liver Diseases?

Most of studies have been performed in patients infected with HCV, and only FT have been investigated specifically in the four most frequent chronic liver disease.

For FT, meta-analysis demonstrated that the diagnostic value of FT was similar in the four most frequent chronic liver diseases. The number of validations must be increased in ALD. We recently demonstrated that the standardization according to the prevalence of fibrosis stages defining advanced and nonadvanced fibrosis is mandatory for any interpretation of AUROCs [29]. For instance, this method allowed an adjustment to be made in the ObAUROCs of FT according to the cause of liver disease, which had significant difference in fibrosis stage spectrum. In HBV studies, patients had lower difference between advanced and nonadvanced fibrosis stages than in studies of ALD patients. After standardization, the difference between AUROCs was reduced by 2 (0.77 vs 0.88 before and 0.80 vs 0.86 after standardization) [29]. Repeated FT improved similarly to fibrosis as estimated by repeated biopsies during treatment for HCV [45, 46] and for HBV [59–61].

For the other tests, the number of non-HCV patients included was relatively small.

5.6. Are There a Specific "Gray Zone" or an "Inaccurate Zone" Between Intermediate Stages?

The different meta-analyses also demonstrated that the diagnostic value of FT, as for liver biopsy, was similar between all the adjacent fibrosis stages but without a specific "gray zone" or an "inaccurate zone" between intermediate stages. FT, like biopsy, has lower diagnostic value to discriminate between two adjacent stages than between two extreme stages [11, 13, 14, 17, 28, 29].

The frequent statement "liver biopsy is still needed for definitive staging of intermediate stages" is not evidence based [106, 107]. The entire liver is certainly the gold standard, but a liver biopsy of 15 mm (the median biopsy length in tertiary centers) has an AUROC of 0.82 between F1 and F2, being around 20% of false positives/negatives [4]. Therefore, FT with an AUROC of 0.66 (usually described as a "weak" value when using a true gold standard) between F1 and F2 has a relative AUROC vs the best AUROC possible of $0.66/0.82 = 0.80$, which is in the end acceptable for a noninvasive test.

The second error is the confusion between intermediate stages and adjacent stages. For any estimate of liver fibrosis, the diagnostic values (AUROCs) between adjacent stages need to be assessed. There are no significant differences in the diagnostic values (AUROCs) for FT [17] or for liver biopsy as demonstrated by Bedossa *et al.* [4] according to intermediate stages as opposed to extreme stages, with the AUROCs for all adjacent stages being similar.

This once again underlines that assessing the AUROCs between all adjacent stages remains the best way, knowing that for the "perfect" biomarker, the best possible achievable AUROC is 0.82 for a 15-mm biopsy.

There are also different methodological approaches for the overview of fibrosis markers. Parkes *et al.* arbitrarily defined an "inaccurate" zone of a marker when it "cannot reliably attribute result for tests as tests perform with lower sensitivities/specificities at thresholds, where positive predictive value is $<90\%$ and negative predictive value is $>95\%$" [108]. There is no rationale for choosing these thresholds, but this definition could be acceptable if a true gold standard existed. This is not the case for fibrosis markers. If this definition is applied to 15-mm liver biopsies, the biopsy will be inaccurate in 40% of cases for a diagnosis between F1 and F2.

5.7. Is the Liver Biopsy Still Useful?

Yes, biopsy is still useful, but not as first-line estimate of liver injury in the four most frequent chronic liver diseases. Biopsy could be useful when validated noninvasive methods, such as FT and FS, are not applicable or discordant. In some countries, like in France, this strategy is already extensively used and approved by health authorities.

Biopsy could be useful when several liver injuries are suspected in the same patients and in less frequent chronic liver diseases. Biomarkers and FS are so far less validated in cholestatic liver diseases (primary biliary cirrhosis and primary sclerosing cholangitis), in patients with liver transplantation, and in very rare disease such as lymphoma or vascular liver disease. In hemochromatosis several algorithms using biomarkers, genetic and imaging tests have been already validated as alternatives to liver biopsy [109].

6. Conclusion

This overview of evidence-based data suggests that biomarkers could be used as an alternative to liver biopsy for the assessment of fibrosis stage in the four more common chronic liver diseases: HCV, HBV, NAFLD, and ALD.

Neither biomarkers nor biopsy are sufficient alone to take definitive decision in a given patient and all the clinical and biological data must be taken into account.

However, due to the dramatically insufficient risk–benefit ratio of biopsy (coefficient variation 40%, 0.3% severe adverse events and 3/10,000 mortality) [4, 9], it is surprising that many leaders and associations in the field of hepatology still recommend liver biopsy as the first-line investigation for millions of people exposed to the risk of fibrosis [11]. Based on current evidence, a wise recommendation would be a moratorium on liver biopsy as a first-line procedure while awaiting studies demonstrating biopsy cost–utility vs that of biomarkers. Biopsy as a second-line estimate of liver injury should still be indicated for intricate diseases or clinicobiological discordances.

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BIOMARKERS RELATED TO AGING IN HUMAN POPULATIONS

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1. Abstract

Biomarkers are increasingly employed in empirical studies of human populations to understand physiological processes that change with age, diseases

0065-2423/08 $35.00
DOI: 10.1016/S0065-2423(08)00405-8

whose onset appears linked to age, and the aging process itself. In this chapter, we describe some of the most commonly used biomarkers in population aging research, including their collection, associations with other markers, and relationships to health outcomes. We discuss biomarkers of the cardiovascular system, metabolic processes, inflammation, activity in the hypothalamic-pituitary axis (HPA) and sympathetic nervous system (SNS), and organ functioning (including kidney, lung, and heart). In addition, we note that markers of functioning of the central nervous system and genetic markers are now becoming part of population measurement. Where possible, we detail interrelationships between these markers by providing correlations between high risk levels of each marker from three population-based surveys: the National Health and Nutrition Examination Survey (NHANES) III, NHANES 1999–2002, and the MacArthur Study of Successful Aging. NHANES III is used instead of NHANES 1999–2002 when specific markers of interest are available only in NHANES III and when we examine the relationship of biomarkers to mortality which is only known for NHANES III. We also describe summary measures combining biomarkers across systems. Finally, we examine associations between individual markers and mortality and provide information about biomarkers of growing interest for future research in population aging and health.

2. Introduction

There is no agreed upon set of biomarkers of aging; however, there is a significant body of literature discussing both what a "biomarker" is and what constitutes aging [1]. These topics are addressed briefly in the beginning of this chapter, but the majority of the chapter focuses on how biomarkers are used in empirical studies of human populations to understand physiological processes that change with age, diseases whose onset appears linked to age, and the aging process itself [2]. We limit ourselves to biomarkers related to general indicators of health and survival that are appropriate for study in human populations *in vivo*, and we do not include biomarkers that are specific to the diagnosis, staging, or prognosis of specific diseases. In our discussion, we indicate the health outcomes that are related to each of the markers, interrelationships between markers, the link between individual and summary biomarkers and mortality, and measures of health used in the older population that are based on multiple indicators. Finally, we indicate future challenges in studying aging populations with biomarkers.

3. Background

3.1. What is a Biomarker?

The lack of an agreed definition for the term "biomarker" was one impetus for the National Institutes of Health (NIH) to recently convene a Biomarkers Definitions working group [3]. The following definition has been offered by this group: "a biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention [4]." In a recent strategic plan for the National Heart Lung and Blood Institute (NHLBI), the word "genotype" was added to the definition before normal biological processes, indicating how the focus of much research has changed since 2001 [5]. The current emphasis on biomarkers arises from an interest in understanding the molecular and physiological basis of disease as well as evaluating therapeutic interventions using surrogate end points rather than death or irreversible disease [6]. Social scientists are interested in adding biomarkers to traditional population studies of health in order to determine how social, psychological, and behavioral factors get under the skin to influence biology and subsequent health outcomes [7, 8].

In populations, biomarkers are used to monitor and predict the health of the population, to identify individuals with particular resistance or susceptibility to health problems, and to evaluate therapeutic interventions. Because of the clinical association of the word "biomarker" with risk factor, one group with a focus on aging populations has used the word "biomeasure" as a higher order term to encompass biomarkers of organic disease, physical condition or function, genetic makers, and biological indicators of aging [9]. In this chapter, we consider all of these types of measures as "biomarkers."

3.2. What is Aging?

While basic scientists continue to try to separate normal aging and disease, scientists interested in population health are more empirically oriented toward defining the age-related health changes that are of interest in evaluating functional ability and survival, which typically represent some combination of aging and disease. Health change in old age has been termed the disablement process by Verbrugge and Jette [10]. In populations, health change occurs in an ordered fashion by age beginning with the development of risk factors, through the onset of diseases and conditions, to functioning loss or loss of ability to perform certain physiological functions and to the onset of disability which is often indicated by inability to work, to care for oneself, or to perform the activities necessary for independent living among older

populations. Frailty is an emerging concept in the study of health outcomes that is specific to older age [11–13]. It is a downward trajectory in health and ability to perform daily tasks resulting from the accumulation of acute and chronic diseases as well as the physiological decline and dysregulation that accompany the onset of diseases and advanced age [12]. Biomarkers can be indicators of any of these aspects of health change: risk, disease, functioning loss, disability, frailty, or imminent death.

4. Biomarkers

In human populations, the identification of biomarkers for health outcomes has resulted from large-scale community and population studies, such as the Framingham study and the NHANES. The MacArthur Study of Successful Aging was the first large-scale community-based study that provided extensive collection of biomarkers in a home-based setting. Because of increases in scientific knowledge of aging and improvements in technology for collection, a growing number of recent population studies have included biomarkers along with collection of social, economic, and psychological information [14]. We note the details of some of these studies at the end of our discussion of individual biomarkers.

In this section, we outline biomarkers that have been used in research on the health of older populations (Table 1) [15–139]. We describe the markers and why they are important in research on aging. This list represents a selection from a significantly larger number of markers that could be described. Our intent is to provide information on the currently most frequently used measures and to indicate some newer measures that are growing in use.

4.1. Cardiovascular System

We begin with indicators of cardiovascular functioning, as heart disease is the leading cause of death in the older population and one of the most important causes of disability (Table 1). The two indicators of blood pressure are probably the most commonly measured biomarkers: *Systolic blood pressure* (SBP) is the maximum pressure in an artery at the moment when the heart is beating and pumping blood; *diastolic blood pressure* (DBP) is the lowest pressure in an artery in the moments between beats when the heart is resting. High levels of either measurement indicate hypertension. Current guidelines define hypertension as SBP $\geq$140 mm Hg or DBP $\geq$90 mm Hg.

SBP is thought to be more important and predictive of aging health outcomes than DBP. There are strong associations between aging, increased SBP, and cardiac and vascular diseases [140]. Studies have shown the

TABLE 1
BIOMARKERS OF AGING

Biomarkers	Description	Measure	Related Outcomes	Source
Biomarkers of cardiovascular system				
Systolic blood pressure (SBP)	Index of cardiovascular activity: maximum pressure in an artery when the heart is pumping blood throughout the body	Physical exam	Cardiovascular death, stroke, CHD, mortality	[15–17]
Diastolic blood pressure (DBP)	Index of cardiovascular activity: lowest pressure in an artery when the heart is resting	Physical exam	Cardiovascular death, stroke, CHD, mortality	[15–17]
Pulse pressure	Indicator of increased arterial stiffness	Physical exam	Stroke, MI, heart failure, cardiovascular death, overall mortality	[18–20]
Resting pulse rate	Indicator of heart functioning and measure of overall fitness	Physical exam	CHD, mortality	[21]
Total homocysteine (tHcy)	An amino acid that plays a role in lipid metabolism; folic acid and vitamin B break down tHcy	Blood	Cardiovascular, cerebrovascular, and peripheral vascular disease, poor cognitive function	[22–25]
Biomarkers of metabolic processes				
Total cholesterol	Aids in the synthesis of bile acids and steroid hormones	Blood	In middle-age: CHD and all-cause mortality; In older ages: U-shaped relation to death	[26–28]
Low-density lipoprotein (LDL)	Transports cholesterol from the liver to be incorporated into cell membrane tissues	Fasting blood	CHD, atherosclerosis, stroke, peripheral vascular disease	[29–32]

(*continues*)

TABLE 1 (*Continued*)

Biomarkers	Description	Measure	Related Outcomes	Source
Very low density lipoprotein (VLDL)	Transports endogenous triglycerides, phospholipids, cholesterol, and cholesteryl esters	Fasting blood	Atherosclerosis, coronary artery disease	[33–34]
High-density lipoprotein (HDL) cholesterol	Protective cholesterol	Blood	Lower atherosclerotic CVD	[35]
Triglycerides	Fat substance stored for energy use	Fasting blood	Heart attack, CHD, CAD, pancreatitis	[33, 36–38]
Fasting glucose	Measures amount of sugar in blood; indicator of diabetes	Fasting blood	Diabetes, CHD, mortality, poor cognitive function	[39–41]
Glycosylated hemoglobin (HbA1c)	Measures amount of sugar binded to hemoglobin in red blood cells	Blood	Diabetes-related complications (eye, kidney, nerve, CHD, stroke), poor cognitive function	[39, 42, 43]
Body mass index (BMI)	Indicator of the balance between energy intake and energy expenditure	Physical exam	CVD, diabetes mellitus, stroke, mortaity, some cancers, osteoarthritis	[44–47]
Waist-to-hip ratio	Indicator of abdominal obesity	Physical exam	Hypertension, CHD, noninsulin-dependent diabetes, stroke	[48–50]
Leptin	Protein hormone that regulates food intake and energy expenditure	Blood	Diabetes mellitus, metabolic syndrome (abdomninal obesity, dyslipidemia, hypertension, hyperglycemia), atherosclerosis, osteoporosis	[51–57]

Adiponectin	Adipose-specific plasma protein that serves as a measure of insulin sensitivity	Fasting blood	Metabolic syndrome (abdomninal obesity, dyslipidemia, hypertension, hyperglycemia); MI	[58, 59]
Biomarkers of inflammation, immunity, and infection				
C-reactive protein (CRP)	Acute-phase response protein that indicates blood levels of inflammation	Blood	CVD, heart attack, stroke, arthritis, cancer, cognitive, physical decline	[60–65]
Interleukin-6 (IL-6)	Immune system regulator (cytokine) that responds to acute illness or injury	Blood, saliva	CVD, immune disorders, AD, diabetes mellitus, certain cancers, functional disability	[60, 65–67]
Fibrinogen	Protein produced by the liver that aids in formation of blood clots to stop bleeding	Blood	CVD, mortality, AD, MCI (γ chain)	[40, 60, 68, 69]
Albumin	Protein that transports small molecules into the blood and maintains oncotic pressure	Blood	Heart attack, stroke, functioning decline, mortality, cognitive impairment	[60, 65, 70, 71]
Tumor necrosis factor-α (TNFα)	Proinflammatory cytokine that stimulates immune and vascular responses	Blood, CSF	Obesity, diabetes, arthritis, stroke	[72–75]
Serum amyloid A (SAA)	Acute-phase protein; main function involves cholesterol transport and lipid metabolism	Blood	CAD, atherosclerosis, cancer, carotid intima medial thickness, depression, obesity	[76–81]
Cytomegalovirus (CMV)	Herpesvirus infectious agent that triggers the immune system	Blood	Dementia, retinal, and gastrointestinal disease	[82, 83]

(*continues*)

TABLE 1 (*Continued*)

Biomarkers	Description	Measure	Related Outcomes	Source
Epstein-Barr virus (EBV)	B lymphotropic herpesvirus; marker of cell-mediated immune function	Blood, saliva	Cancer infectious mononucleosis	[84–86]
T cells	White blood cells that protect against pathogens and tumors	Blood	Cancer, mortality, artherosclerosis, AD	[87]
Biomarkers of the central nervous system				
Amyloid β42	Major component of senile plaques	CSF	Inverse relation to neuropathological processes (AD); frontotemporal and vascular dementia	[88–91]
Total (t)-Tau	Major protein constituting neurofibrillary tangles	CSF	AD; Creutzfeldt-Jakob disease	[89, 92]
Phosphorylated (p)-Tau	Precedes formation of neurofibrillary tangles	CSF	AD, MI	[91, 93, 94]
F2-isoprostanes (F2-iso)	Isomer of prostaglandins stored in cells; stable, free radical-catalyzed products that reflect lipid peroxidation	CSF	AD, hypercholesterolemia, atherosclerotic plaque	[95–99]
Biomarkers of the HPA and the sympathetic nervous system				
Cortisol	Steroid hormone that reflects body's response to physiological stress	Blood, saliva, urine	CVD, poor cognitive functioning, fractures, functional disability, mortality	[100–104]
Dehydroepiandrosterone sulfate (DHEA-S)	Antagonist of cortisol; steadily decreases with age	Blood, saliva, urine	Inverse relation to heart disease, mortality, physical, and mental functioning, AD	[105–111]

Free insulin-like growth factor-1 (IGF-1)	Growth factor that regulates cell growth and development; Inhibitor of programmed cell death	Fasting blood	Cancer; inverse relation to atherosclerotic plaques, CAD, osteoarthritis, mortality	[112–115]
Norepinehrine	Indicator of stress response	Blood, urine	CHF, MI, mortality	[116–118]
Epinephrine (adrenaline)	Stress hormone important to body's metabolism; prepares for strenuous activity of the "fight or flight" response	Blood, urine	Cognitive decline and possibly poor survival with prior MI	[119–121]
Biomarkers of organ function				
Creatinine	In clinical practice, an index of renal function	Blood, urine	Cardiovascular risk, renal diseases, mortality	[122–125]
Cystatin C (CysC)	Detects rapid glomerular filtration rate	Blood	Acute renal failure, diabetic nephropathy, thyroid dysfunction, mortality	[126–131]
Peak expiratory flow (PEF)	Measurement of airway obstruction	Spirometry exam	Asthma, chronic obstructive pulmonary disease	[132, 133]
Electrocardiogram (EKG)	Measurement of electrical impulses in the heart	Physical exam	Cardiovascular risk, stroke, mortality	[134–136]
Biomarkers of oxidative stress				
Reactive oxidative species (ROS)	Involved in programmed cell death and apoptosis, induction of host defense, mobilization of ion transport systems	Blood	Parkinson's disease, DNA damage (cancer)	[137, 138]
Superoxide dismutase (SOD)	Important antioxidant defense in cells exposed to oxygen	Blood	Inverse relation to AD	[139]

CHD=Coronary heart disease; AD=Alzheimer's disease; MI=myocardial infarction; CAD=coronary artery disease; CVD=cardiovascular disease; MCI=mild cognitive impairment; PD=Parkinson's disease; CHF=congestive heart failure; CSF=cerebrospinal fluid.

stronger predictive power of SBP for coronary heart disease (CHD) and life expectancy at advanced ages [27, 141, 142]. Among the Framingham Heart Study participants, SBP was directly related to CHD risk, but DBP was inversely related to the risk in older ages (60+) [143].

Pulse pressure (PP) is an alternative measure indicating the difference between the SBP and DBP that some researchers prefer for use in studying the aged. The rise in SBP and PP in middle-aged and elderly subjects is mainly related to increased large-artery stiffness and an associated increase in wave reflection amplitude [144]. Increasing evidence shows that PP predicts risk of CHD in middle and old ages [19, 143, 145]. During middle age, SBP and DBP change similarly; however after age 60, DBP decreases and SBP continues to rise resulting in the large increase in PP in old ages [143]. While factors such as smoking, lack of physical activity, and drinking affect PP, studies have shown the independent effect of PP on health outcomes after adjusting for such risk factors [146].

Heart rate, considered one of the four vital signs, is based on the number of heartbeats per minute (bpm). In most cases, the pulse is an accurate measure of heart rate, and the two terms are often used synonymously; although in individuals with certain arrhythmias, heart rate and pulse rate may not be equivalent. *Pulse rate* is commonly measured from the brachial artery (the wrist) or the carotid artery (the neck).

Since pulse rate increases with physical exercise, it is commonly measured during resting, nonphysical exertion conditions. At rest, the average adult pulse rate is 70 bpm for males and 75 bpm for females; however, these rates may vary by age, sex, race and ethnicity, and exercise status. At birth, pulse rate ranges from 100 to 180 bpm and gradually decreases to range from 60 to 110 bpm until age 16 [21, 147]. Between ages 25–74, no consistent changes in pulse rate with age have been found [148]. Gender and racial differentials indicate that women have higher resting pulse rate than men and White women have higher pulse rates than Black women [148]. Finally, athletes exhibit much lower resting pulse rates as a result of strengthened heart muscle from regular exercise [149].

A pulse rate of 90 bpm or greater is considered high [150] and is associated with increased risk of CHD, as well as cardiovascular, noncardiovascular, and all-cause mortality [21, 151]. Consequently, both medical (e.g., medication) and nonmedical modifications (e.g., life style modifications including increases in physical activity and lower fat diets) can reduce resting pulse rate, and, in turn, reduce the risk of cardiovascular disease and mortality [152–154].

All of the above markers are collected in a physical exam. There are many other biomarkers linked to cardiovascular risk that are determined in other ways. One of these is *homocysteine*, an amino acid measured from blood plasma. Homocysteine affects the development of atherosclerosis by

damaging the inner lining of arteries and promoting blood clots. For this reason, we are including it with other cardiovascular risk factors even though it differs from the others in that it is measured with blood. Homocysteine has garnered recent attention because of its importance in predicting many of the major health outcomes common in aging populations, including cardiovascular disease, peripheral vascular disease, and poorer cognitive function [22–25]. It is highly related to dietary content including folate and vitamins B_{12} and B_6 [155, 156]. In the early 1990s, approximately one-third of those older than 65 years had elevated homocysteine levels (>14 μmol/liter) [157]; however, the prevalence has declined markedly since dietary fortification with folate began in 1996 [156, 158].

We indicate the interrelationships among the cardiovascular biomarkers for a nationally representative sample of persons aged 65 and over in the NHANES and for the MacArthur Sample of Successful Aging participants who were aged 70–79. Biomarkers are dichotomously defined using the level of each biomarker to classify sample members into those at a level defined as at clinical risk, or in the top quartile of the sample, or not. Clinical risk levels are shown in Table 2 [159–171] and the phi coefficients among cardiovascular markers in Table 3. A phi coefficient is a measure of the degree of association between two binary variables and is interpreted like a pearson correlation coefficient. The most significant coefficients are between SBP and DBP, which are moderately related with a coefficient of 0.19 (NHANES) and 0.34 (MacArthur), and between SBP and PP, which are relatively strongly related with a correlation of 0.48 (NHANES). With the exception of SBP, DBP, and PP, high risk levels of these biomarkers occur fairly independently of each other.

4.2. Markers of Metabolic Processes

The next set of markers is indicators of metabolic processes, many of which are also related to cardiovascular outcomes. *Cholesterol* has several functions including keeping cell membranes intact and helping the synthesis of steroid hormone and bile acids. In recent years, components of total cholesterol are generally measured to determine risk for heart disease: *low-density lipoprotein* (LDL), *high-density lipoprotein* (HDL), and *very low density lipoprotein* (VLDL) [172]. In middle-aged populations, total cholesterol level has been shown to have a direct relation with CHD and all-cause mortality [26]. However, in older persons, the relationship between cholesterol and mortality has been found to be U- or J-shaped [27, 28]. Comorbidity may need to be considered in evaluating the risk implied by cholesterol levels among frail older persons [17, 173, 174].

TABLE 2
CLINICAL OR EMPIRICALLY DERIVED CUTOFFS FOR RISK FACTORS

Biomarkers	High risk cutpoints	Source
Biomarkers of cardiovascular system		
Systolic blood pressure	≥140 mm Hg (N)	[159]
	≥148 mm Hg (M)	[160]
Diastolic blood pressure	≥90 mm Hg (N)	[159]
	≥83 mm Hg (M)	[160]
Pulse pressure	≥88 mm Hg (N)	NHANES III 1999–2002 fourth quartile[a]
Resting pulse rate	≥90 bpm (N)	[150]
Homocysteine	≥15 μmol/liter (N)	[161, 162]
	≥13.38 μmol/liter (M)	[163]
Biomarkers of metabolic processes		
Serum total cholesterol	≥240 mg/dl (N)	[164]
Serum HDL cholesterol	≥40 mg/dl (N)	[164]
	≥37 mg/dl (M)	[160]
Total/HDL cholesterol	≥5.92 (M)	[160]
Serum LDL cholesterol	≥160 mg/dl (N)	[164]
Serum triglycerides	≥200 mg/dl (N)	[164]
Fasting blood glucose	≥126 mg/dl (N)	[164]
Glycosylated hemoglobin	≥6.4% (N)	[164]
	≥7% (M)	[160]
Body mass index	≥30 kg/m^2 (N)	[166]
	≥28.59 kg/m^2 (M)	[163]
Waist-to-hip ratio	≥0.94 (M)	[160]
Serum leptin	≥17.2 μg/liter (N)	NHANES III (1988–1994), fourth quartile[a]
Biomarkers of inflammation		
C-reactive protein	≥3 mg/liter (N)	[167]
	≥3.19 mg/liter (M)	[160]
IL-6	≥4.64 pg/ml (M)	[160]
Plasma fibrinogen	≥400 mg/dl (N)	[168]
	≥336 mg/dl (M)	[160]
Albumin	<3.8 g/dl (N)	[169]
	≤3.9 g/dl (M)	[160]
Biomarkers of HPA and SNS		
Urinary cortisol	≥25.69 μg/g creatinine (M)	[160]
DHEA-S	≤350 ng/ml (M)	[160]
Norepinephrine	≥48 ug/g creatinine (M)	[160]
Epinephrine	≥4.99 ug/g creatinine (M)	[160]
Markers of organ functioning		
Creatinine clearance	<30 ml/min (N)	[170]
	≤44.64 ml/min (M)	[160]
Best peak flow	<550 liter/min (males) (N)	NHANES III (1988–1994), fourth quartile[a]
	<400 liter/min (females) (N)	
	≤300 liter/min (M)	[160]
Cystatin C	>1.55 mg/liter (N)	[171]

(N) NHANES; (M) MacArthur.

HDL = high-density lipoprotein; LDL = low-density lipoprotein; IL-6 = interleukin-6; DHEA-S = dehydroepiandrosterone sulfate.

[a] Individual data from NHANES III (1988–1994), using the highest quartile as at risk.

TABLE 3
PHI COEFFICIENTS AMONG HIGH RISK LEVELS OF CARDIOVASCULAR BIOMARKERS

(a) Ages 65+ in the NHANES 1999–2002 (N=1,884)

	DBP	SBP	Pulse pressure	Resting pulse rate	Homocysteine
DBP		0.19***	−0.04+	−0.01	−0.01
SBP			0.48***	−0.02	0.03
Pulse pressure				−0.03	0.05+
Resting pulse rate					0.07*
Homocysteine					

(b) Ages 70–79 in the MacArthur Study of Successful Aging [N=654 (N=363 for correlations to homocysteine)]

	DBP	SBP	Homocysteine
DBP		0.34***	0.09
SBP			−0.03
Homocysteine			

DBP = diastolic blood pressure; SBP = systolic blood pressure.
***p <0.0001, **p <0.001, *p <0.01, +p <0.05.

LDL is sometimes referred to as "bad" cholesterol because elevated levels of LDL correlate most directly with CHD [32]. Current guidelines indicate that a desirable level of LDL cholesterol is below 130 mg/dl; borderline high is from 130 to 159 mg/dl; high is between 160 and 189 mg/dl; and very high LDL-cholesterol is $\geq$190 mg/dl. Recently, recommended target levels of cholesterol were adjusted to be lower for those with diabetes and other heart disease risk factors. Those who have established coronary disease and diabetes have a recommended target for an LDL cholesterol level less than 70 mg/dl [29]. Generally, a high level of LDL cholesterol has been shown to contribute to the development of coronary atherosclerosis and to increased risk of mortality and heart disease [175]; however, studies limited to older persons have shown inconsistent findings on the relationship between LDL and health outcomes [40, 173, 176–183].

Akin to LDL, levels of VLDL increase with age and are also commonly referred to as "bad" cholesterol [184]. While VLDL is not measured as frequently in population studies, it may be a better indicator of risk in older people. Among individuals aged 50 or older, VLDL was a better predictor of the development of coronary artery disease, while LDL cholesterol was more significant among people under age 50 [34].

High levels of HDL are protective for heart disease because HDL carries cholesterol away from the arteries and back to the liver, where it is passed from the body. Thus, HDL is called the "good" cholesterol and low levels are associated with higher risk. HDL cholesterol levels less than 40 mg/dl

(although sometimes this level is sex specific) have been related to increased risk for heart disease [185–187].

While traditional lipid measures, such as total cholesterol and HDL, are often used independently to indicate lipid profiles and their relations to health outcomes, studies have shown that *total cholesterol/HDL ratio* can be used as a biomarker that is associated with other cardiovascular risk factors [188, 189] and predicts ischemic heart disease risk [190] and atherosclerotic plaque rupture [191].

Triglycerides, an indicator of stored fat, are often included among the lipid indicators as part of an evaluation of coronary risk factors. Normal fasting triglyceride levels are below 150 mg/dl; 150–199 mg/dl is considered borderline high, 200–499 mg/dl high, and 500 mg/dl and above very high [33]. High triglyceride levels have been associated with heart attack [192], CHD [36], and coronary artery disease [37].

Tests for total cholesterol, LDL, HDL, and triglycerides are routinely done in lipid panels. Accurate results for the entire lipid panel assume 9–12 hours of fasting; however, total and HDL cholesterol can be measured without fasting and thus are more likely to be included in assays from large population surveys without fasting subjects. Fasting is required for valid results for LDL, VLDL, and triglycerides.

Fasting blood glucose level is indicative of diabetes and prediabetes. Higher than normal blood glucose contributes to the development of metabolic syndrome and CHD [193, 194]. About 11.9 million adults in the United States aged 45–74 had prediabetes levels of glucose in the year 2000 and this included a quarter (22.6%) of overweight adults [195]. A normal blood glucose level is between 70 and 99 mg/dl. A fasting blood glucose level between 100 and 125 mg/dl signals prediabetes and a higher level indicates diabetes [196].

Because it can be collected in a nonfasting sample, many researchers are measuring *glycosylated hemoglobin* (HbA1c) as an alternative to fasting glucose for diabetes screening [197]. The percentage of glycosylated cells increases with more glucose in the blood and provides an indicator of the amount of sugar that is attached to the hemoglobin in red blood cells. Because red blood cells live in the bloodstream for approximately 4 months, the HbA1c test shows the average blood sugar for the past 2–3 months and is an indicator of glucose metabolism over that time. Results of this test can indicate prediabetes and are used in managing diabetes. HbA1c levels have been related to cardiovascular disease and mortality among both diabetics and nondiabetics [198] and to CRP levels [199]. Some studies show age-related increases in HbA1c [200, 201], while others show little or no age-related increase in HbA1c [202], possibly due to its relationship to mortality.

Anthropometric measures such as *weight, body mass index* (BMI), *waist and hip circumference*, and *waist-to-hip ratio* (WHR) can all be used to

indicate weight and adiposity. BMI is calculated as the ratio of weight to height-squared (kg/m^2). Overweight is defined as a BMI between 25 and 29.9 kg/m^2 and obesity as BMI $\geq$30 kg/m^2 [160]. However, the validity of BMI as a measure of excess fat declines in older people because of height loss and increases in fat mass occurring with age even in the absence of weight gain. Some researchers prefer WHR and waist circumference (WC) to BMI as a predictor for cardiovascular risk [102] and other adiposity-related conditions. While BMI provides an index of obesity, WHR may be more useful as an index of chronic metabolic dysregulation and adipose tissue deposition [203]. Researchers have argued that it is not obesity *per se* but the distribution of the adipose tissue that is related to increased risk [204, 205]. Those with an apple body shape or a central distribution of fat tend to experience higher rates of atherosclerotic heart disease, stroke, hypertension, hyperlipidemia, and diabetes than those with a pear body shape. According to the guidelines for defining metabolic syndrome [3], the use of a simple measure of WC instead of BMI is recommended to identify the body weight component of metabolic syndrome (men >40 in.; women >35 in.).

Those with higher values of BMI, waist and hip circumferences, and WHR tend to be at higher risk for hypertension, adult-onset diabetes mellitus, heart disease, stroke, various forms of cancer, atherosclerosis [44, 45, 47, 205–209], osteoarthritis [46], lower aerobic capacity and less muscle strength [210], and disability [211–215].

Leptin is a hormone that plays an important role in the long-term regulation of body weight. As a crucial regulator of food intake and energy balance, leptin is involved in the physiology of various diseases. In old age, declines in organ function and changes in hormone secretion result in the alteration of leptin secretion [216]. Although it is uncertain whether aging has an independent effect on leptin levels, it is known that some changes common in old age (e.g., declines in bone turnover and slower rates of glucose and lipid metabolism) are related to leptin levels. Studies have indicated that leptin may play an important role in several chronic diseases, including metabolic syndrome, atherosclerosis, malnutrition, diabetes mellitus, dyslipidemia, hypertension, osteoarthritis, and osteoporosis [54–57].

Examination of the interrelationships of risk levels among the metabolic markers available in the NHANES data indicates that total and LDL cholesterol are highly related (0.76) in the fasting population (Table 4a). Neither high risk levels of total or LDL cholesterol are very highly related to high risk levels of HDL cholesterol. HDL risk is moderately highly related to having high triglycerides (0.24), fasting blood glucose (0.15), and glycated hemoglobin (0.15). High-risk leptin levels are strongly related to high BMI (0.40). High BMI is moderately related to fasting blood glucose (0.12) and HbA1c (0.11), but not very closely related to any of the cholesterol indicators. The

TABLE 4
PHI COEFFICIENTS AMONG HIGH RISK LEVELS OF METABOLIC BIOMARKERS

(a) Ages 65+ in the NHANES 1999–2002 (NHANES III for Leptin) (N=1,884 for nonfasting biomarkers, N=938 for fasting biomarkers; N=2741 for nonfasting biomarkers, N=1172 for fasting biomarkers for NHANES III)

	Cholesterol	HDL	LDL[a]	Triglycerides	Blood glucose[a]	Glycosylated hemoglobin	BMI	Leptin[a,b]
Cholesterol		−0.08***	0.76***	0.09*	0.02	0.06*	0.01	0.09*
HDL			−0.05	0.24***	0.15***	0.15***	0.06*	−0.10**
LDL[a]				0.10*	−0.02	−0.04	0.00	0.04
Triglycerides[a]					0.20***	0.12***	0.10**	0.10*
Blood glucose[a]						0.67***	0.12***	0.04
Glycosylated hemoglobin							0.11***	−0.00
BMI								0.40***
Leptin[a,b]								

(b) Ages 70–79 in the MacArthur Study of Successful Aging (N=654)

	Cholesterol/HDL	HDL	Glycosylated hemoglobin	BMI	Wasit/Hip
Cholesterol/HDL		0.55***	0.09+	0.02	0.11*
HDL			0.06	0.03	0.16***
Glycosylated hemoglobin				0.13**	0.09+
BMI					0.20***
Waist/hip					

HDL = high-density lipoprotein; LDL = low-density lipoprotein; BMI = body mass index.
*** p <0.0001, ** p <0.001, * p <0.01, + p <0.05.
[a] Fasting biomarkers: LDL, triglycerides, blood glucose, leptin.
[b] Correlations to leptin are based on biomarkers from NHANES III.

relationships among indicators of metabolic risk for the MacArthur data are shown in Table 4b. Analyses of these data have included total/HDL cholesterol ratio and the WHR among metabolic indicators [160]. Again, most of the relationships among the indicators are modest.

4.3. Markers of Inflammation, Immunity, and Infection

Markers of Inflammation are the next category of markers. Age-related changes in inflammatory markers are complex and include a wide range of potential indicators. Here we focus on the markers most commonly used in aging research. *C-reactive protein* (CRP) is an acute phase response protein produced in the liver that indicates general systemic levels of inflammation. CRP levels rise as part of the immune response to infection and tissue damage or injury and may be elevated due to the presence of chronic conditions, like diabetes, asthma, rheumatoid arthritis, and heart disease [61, 217–221]. In an acute response, the level of CRP can jump a thousand-fold but then drops relatively quickly when an infection passes. A blood level above 10 mg/dl is considered indicative of acute illness, although recent work has shown this level to be related to chronic conditions such as obesity and poor social conditions (e.g., living in poverty) [222]. CRP levels are also related to hormone levels in women and are elevated with the use of oral contraceptives or postmenopausal hormone replacement therapy.

Research has suggested that high levels of CRP, between 3 and 10 mg/dl [223], are related to the development of cardiovascular disease [61, 221, 224, 225] and cardiac events, including heart attack [60] and stroke [61]. This level of CRP has also been related to mortality [64, 65] and physical decline [65]. In contrast to many clinical settings, researchers use what is called a high-sensitivity CRP test (hs-CRP) to determine moderate (1–3 mg/dl) as well as higher levels of CRP. Hs-CRP can be measured with whole blood samples or blood spots [226].

Interleukin-6 (IL-6) is one of a class of immune system regulators called cytokines that serve a variety of immune functions in response to acute illness or injury and is perhaps the most commonly measured cytokine in population surveys. As a pro-inflammatory cytokine, IL-6 is involved in activating inflammatory pathways. IL-6 is always present in the body in small amounts ($<$1–2 μg/ml), and its concentration varies by time of day. However, in periods of immune activation, blood levels of IL-6 increase quickly, reaching as high as 40 times normal levels. IL-6 levels also rise with advancing age and are related to a variety of chronic conditions. The dysregulation of IL-6 may be a contributing factor to many of the diseases of aging.

Chronic conditions associated with high IL-6 include osteoporosis, arthritis, type-2 diabetes, certain cancers, and Alzheimer's disease (AD) [66, 67].

High levels of IL-6 are also related to cardiovascular disease, heart attack, and stroke [60, 227–232]. In the elderly, high IL-6 levels are related to an increased risk of functional disability and functional decline [65, 70, 233], cognitive decline [234], and mortality [64, 65]. The association of IL-6 with cardiovascular disease is related to the central role this cytokine plays in promoting the production of CRP [60, 235]. Blood serum sample is required for IL-6 assays.

While less commonly included in large-scale studies, several *other inflammatory cytokines* have been linked to age-related outcomes. For instance, IL-10 is a pro-inflammatory cytokine also important to inflammatory and immunological responses [236]. IL-6 soluble receptor (IL-6sR) is important in the transition from acute to chronic inflammatory states [237]. IL-1β mainly stimulates T-helper cells that secrete IL-2, a cytokine that supports the proliferation of inflammatory cells [238] and influences the function of other cells by binding to IL-1 receptor antagonist (IL-1ra). IL-18, formerly called interferon (IFN)-γ inducing factor (IGIF), is closely related to IL-1. It induces IFN-γ produced in T cells, natural killer (NK) cells, gene expression, and the synthesis of *tumor necrosis factor*-α (TNFα) [239] (further described below). The cascade of inflammatory markers is highly interrelated and complex. Age-related increases in many of the cytokines have been noted [240], but further research on the associations of these individual markers is required before it is clear which can be included most usefully in population studies. But development of assays that can simultaneously measure a large number of inflammatory markers in dried blood spots is likely to increase markers measured in populations [241].

Fibrinogen, also called serum fibrinogen, plasma fibrinogen, and factor I, is a protein produced by the liver. Fibrinogen helps stop bleeding by promoting the formation of blood clots. Fibrinogen has been shown to be strongly predictive of both mortality [40] and the onset of cardiovascular disease [60, 227, 231, 242]. The relationship between socioeconomic status and fibrinogen levels has been suggested as a mechanism linking low social status and stress to cardiovascular disease [243–246]. Fibrinogen is measured using blood serum or plasma.

Albumin is a protein that transports small molecules in the blood and is important in maintaining oncotic pressure in the blood. Low albumin may be related to malnutrition or a low-protein diet and liver or kidney disease. Low albumin levels can also be related to inflammation. For this reason, albumin is sometimes included in indices of inflammation [247]. Low levels of albumin have been related to heart attack, stroke, functioning loss, and death among older persons [60, 65, 70, 227–233]. Data from the MacArthur study have related low levels of albumin to functional decline, death [65], and cognitive impairment [71]. Concomitant low serum cholesterol and albumin levels may

identify high-functioning older persons who are at increased risk of subsequent mortality and functional decline [248]. The test for albumin levels requires blood serum. In the MacArthur Study of Successful Aging analysis of allostatic load, low albumin has been included as a risk factor with a cutoff of 3.9 mg/dl or lower considered as high risk [160].

TNFα is a pleotrophic polypeptide that plays an important role in inflammation and immune function. Expression of TNFα correlates with the expression of other cytokines, including IL-6 and IL-1. Mounting scientific evidence suggests that elevated blood plasma TNFα concentration is associated with dementia in centenarians [249] and is centrally involved in the pathogenesis of AD [250–255]. Additionally, high levels of TNFα are related to atherosclerosis [256], obesity and diabetes [72, 73], rheumatoid arthritis [74], and stroke [75].

Serum amyloid A (SAA), a grouping of acute-phase proteins, increases dramatically in response to injury and inflammation [257]. These proteins transport cholesterol to the liver for bile secretion, recruit immune cells to sites of inflammation, and induce enzymes to degrade extracellular matrix [76]. SAA is involved in chronic inflammatory diseases (e.g., atherosclerosis, coronary artery disease, and rheumatoid arthritis) [77–79, 258], and it is linked to lung cancer, depression, and obesity [78, 80, 81].

Interrelations among the inflammatory markers available in the NHANES and MacArthur studies are shown in Table 5a and b. High risk levels of fibrinogen and high risk CRP are relatively strongly related in both studies (0.33). High risk CRP and high risk IL-6 are also relatively strongly related (0.37) in the MacArthur study. There is a small relationship between high

TABLE 5
PHI COEFFICIENTS AMONG HIGH RISK LEVELS OF MARKERS OF INFLAMMATION

(a) Ages 65+ in the NHANES 1999–2002 (N=1,884)

	CRP	Fibrinogen	Albumin
CRP		0.33***	0.11***
Fibrinogen			0.09***
Albumin			

(b) Ages 70–79 in the MacArthur Study of Successful Aging (N=654)

	CRP	IL-6	Fibrinogen	Albumin
CRP		0.37***	0.33***	0.06
IL-6			0.19***	−0.01
Fibrinogen				0.04
Albumin				

CRP = C-reactive protein; IL-6 = interleukin-6.
$^{***}p < 0.0001$, $^{**}p < 0.001$, $^{*}p < 0.01$, $+p < 0.05$.

risk albumin and high risk CRP (0.11), between high risk albumin and high risk fibrinogen (.09) in NHANES, but not in MacArthur study.

The next set of markers is indicative of the functioning of the immune system.

Cytomegalovirus (CMV) is a herpesvirus that infects most people relatively early in life. The prevalence of CMV infection within the US population increases with age reaching 91% in people ages 80 and over [259–261]. It has been suggested that CMV is a "driving force" behind age-related changes in T cells [262–266]. The proposed CMV-driven pathway occurs through an increase in CMV-specific CD8+ T cells that, in turn, lead to a reduction in the immune system's ability to respond to other infectious pathogens. CMV seropositivity and high antibody levels have been associated with inflammation, cardiovascular disease, stroke, endothelial dysfunction, frailty, and cognitive decline [267–271].

Epstein-Barr virus (EBV) is another common herpesvirus that affects most people during their life. The prevalence of EBV is as high as 95% among adults between ages 35 and 40 in the United States. EBV antibody level is used by some researchers as a marker of cell-mediated immunity [272–278]. The pattern of significantly higher EBV levels at older ages is suggestive of some loss of cellular immunity in older age [272].

T-helper cells, also known as CD4 or T4 cells, are white blood cells that are a major component of the immune system. CD4 count assesses the status of the immune system. A normal CD4 count in adults ranges from 500 to 1350 cells per cubic millimeter (mm^3) of blood. A count of 250–350 CD4 cells/mm^3 suggests some immune system damage and less than 200 CD4 cells/mm^3 is often indicative of more serious immune system damage [279].

In addition to its value as an indicator of a compromised immune system, the CD4 count has been used in the measurement of age-related changes in the immune system [280–282]. The CD8 count has also been associated with age-related conditions. High circulating levels of CD8 T cells have been associated with chronic infections, including EBV and CMV [283]. CD8+ T cells respond to chronic systemic intracellular pathogens whereas CD4+ T cells respond to specific extracellular pathogens. A constant CD4:CD8 ratio indicates healthy aging, while a decline in this ratio can indicate increased immunological risk in the elderly [284].

4.4. Markers of the Central Nervous System

Many potentially useful biomarkers are obtained via obtrusive or invasive measures and are not currently collected in large population studies. For instance, several potential markers for AD from cerebrospinal fluid (CSF) have been proposed but are not collected. *Amyloid β42* is a major component

of senile plaques and is a suggested marker of neuropathological processes related to AD [88, 89]. *Total (t)-tau* is a major protein that comprises *neurofibrillary tangles*, and *phosphorylated (p)-tau* precedes formation of neurofibrillary tangles. High CSF levels of both t-tau and p-tau are associated with an increased risk of AD [89–91]. Additionally, *F2-isoprostanes* (F2-iso) are prostaglandins that reflect lipid peroxidation. F2-iso are associated with AD, hypercholesterolemia, and atherosclerotic plaque [95–99]. Although several studies have used or are currently using these indicators as markers of AD, collection of CSF is not feasible for large population studies.

4.5. Markers of Activity in the Hypothalamic Pituitary Axis

Cortisol is a steroid hormone produced by the adrenal cortex in response to internal or external stress [289]. Consistently high cortisol reactivity to repeated challenges is an atypical response that may reflect chronic physiological stress [285] and is associated with negative health outcomes in old age [286]. Cortisol and its antagonist, dehydroepiandrosterone sulfate (DHEA-S) (described below in more detail), are indicators of HPA activity. Cortisol has a strong diurnal variation, generally high early in the morning and falling during the day [287]. Cortisol typically increases over the first few minutes of the day, reaching a peak 20–30 min after waking.

Cortisol levels have been shown to be greater among individuals experiencing chronic stress from work or emotional strain [288]. Health consequences of exposure to elevated cortisol include increased cardiovascular risk [100], poorer cognitive functioning [101, 286], and increased risks for fractures [103].

Cortisol level can be assessed using blood, saliva, and urine. Urine is collected over a 12- or 24-hour period in order to represent a daily level [286]. Researchers are often interested in the profile of cortisol change over the day; including the rise in cortisol levels after waking in the morning. For this reason, salivary cortisol may be measured four or five times in the same day—upon waking, shortly afterward, in the afternoon, evening, and night [287]. Normal levels of cortisol in the bloodstream range from 6 to 23 μg/dl. Normal 24-hour urinary cortisol levels range from 10 to 100 μg per 24 hours [288]. In the MacArthur study, the level used to define risk for urinary cortisol was $\geq$25.69 μg/g creatinine [160].

DHEA is a hormone produced by the adrenal gland. *DHEA-S* is synthesized from DHEA and converted into other hormones [290]. Assays measure DHEA-S instead of DHEA because DHEA-S is less rapidly cleared from the bloodstream and has less diurnal variation [290–293]. DHEA-S has been hypothesized to serve as a functional antagonist to HPA activity and thus is an important indicator of overall activity in the HPA [294–302].

The level of DHEA is age related. Production of DHEA stops at birth, then resumes around age 7 and peaks when people are in their mid-twenties. From the early thirties on, there is a steady decline (about 2% each year) until around age 75, when the level of DHEA in the body is about 5% of the peak level. Because DHEA-S is related to age and longevity [296–302], it has attracted attention for possible "antiaging" effects [303–305]. Normal values for serum DHEA-S vary with sex as well as age. Normal ranges are 800–5600 μg/liter for men, 350–4300 μg/liter for women; although there may be slight variation in these levels across laboratories. DHEA assays can be based on blood, saliva, or urine samples.

While there are mixed results by gender [306], the literature generally documents a link between low DHEA-S and poor health outcomes. Lower DHEA-S is related to a history of heart disease and mortality [105–108]. DHEA-S is hypothesized to be protective against heart disease because of its anticlotting and antiproliferative properties [106, 307]. Low DHEA-S has also been related to worse physical and mental functioning [109, 110, 308]. Low DHEA-S has been included as one component of allostatic load [102, 309]. In addition, studies have found that DHEA-S is a marker for bone turnover predicting bone mineral density [310], and low levels have been linked to AD [111, 295].

Insulin-like growth factor-1 (IGF-1) is a polypeptide protein hormone that modulates cell growth and survival. Throughout the lifespan, IGF-1 impacts neuronal structure and function, mainly through its effects on growth hormone (GH) [311]. A meta-analysis indicated that high IGF-1 concentrations are associated with increased risk of prostate cancer and premenopausal breast cancer [112]. Conversely, low IGF-1 levels have been linked to increased mortality [114, 312, 313], coronary artery disease [113], and osteoarthritis [114]; however, a recent study on the nationally representative NHANES sample showed no relationship between low IGF-1 and all-cause mortality or mortality from heart disease or cancer [314].

4.6. Markers of the Sympathetic Nervous System

Norepinephrine is a neurotransmitter in the catecholamine family, which mediates chemical communication in the SNS. Norepinephrine is almost identical in structure to epinephrine, another catecholamine discussed below. Both of these are indicators of a stress response. With advancing age, there is decreased clearance of norepinephrine [118] and normal aging is associated with an increase in plasma norepinephrine levels [315–317]. High plasma norepinephrine levels have been associated with increased overall mortality in the elderly [29] as well as reduced survival in healthy older

persons, in patients with congestive heart failure [116], and in people with previous myocardial infarction (MI) [117]. Higher levels of urinary catecholamine excretion have also been shown to predict functional disability and mortality [104].

Norepinephrine is excreted in urine and 12-hour or 24-hour urine collections are used for daily levels because levels vary over the day. To adjust for body size, results for norepinephrine are reported as micrograms norepinephrine per gram creatinine of urine excretion [104, 120]. There are no normative values for urinary norepinephrine and epinephrine levels so adverse catecholamine levels have been classified as those in the top tertile or top quartile of norepinephrine for a sample. In the MacArthur study, the risk level cutoff was 48.00 μg/g creatinine. A blood plasma test is also available although used more rarely.

Epinephrine is another stress hormone, also known as adrenaline. Heightened secretion caused by fear or anger is part of the ''fight or flight'' response and is linked to increased heart rate and the hydrolysis of glycogen to glucose. Increases over time in urinary excretion of epinephrine predict subsequent cognitive decline in older men [120]. High plasma epinephrine has been associated with poor survival in patients with previous MI [121] but increased survival among healthy older persons [119]. Urinary epinephrine excretion is significantly lower among women and among subjects with a BMI >27 kg/m^2. Current smokers have higher levels of both urinary norepinephrine and epinephrine [104].

Measurement of epinephrine is similar to that of norepinephrine: usually in urine from 12-hour or 24-hour urine collections, adjusted for body size by reporting epinephrine per gram creatinine of urine excretion [104, 120]. Like norepinephrine, there are also no normative values for urinary epinephrine levels, and they are generally classified using quartiles or tertiles for

TABLE 6
PHI COEFFICIENTS AMONG HIGH RISK LEVELS OF MARKERS OF SNS AND HPA AGES 70–79 IN THE MACARTHUR STUDY OF SUCCESSFUL AGING (N=654)

	Cortisol	DHEA-S	Norepinephrine	Epinephrine
Cortisol		0.08+	0.01	0.11*
DHEA-S			0.09+	0.06
Norepinephrine				0.27***
Epinephrine				

DHEA-S = dehydroepiandrosterone sulfate.
$^{***}p<0.0001$, ** $p<0.001$, * $p<0.01$, $+p<0.05$.

individual samples. The MacArthur study used a cutoff of greater than 4.99 μg/g creatinine to denote high risk epinephrine. Epinephrine may also be determined from blood plasma assay although this is used more rarely than urinary assays. Like urinary levels, plasma levels of catecholamines may be influenced by a variety of postural, diurnal, and acute stress-related factors [318].

The MacArthur data have four biomarkers indicating SNS and HPA activity (Table 6). Epinephrine is related to norepinephrine with a coefficient of 0.27, and the correlations among other markers are weak or insignificant.

4.7. Markers of Organ Function

Creatinine is a chemical waste molecule generated from muscle metabolism. It is transported through the bloodstream, filtered in the kidneys, and excreted in the urine. It provides information on kidney function. Normal levels of creatinine in the blood are $<$1.5 mg/dl in adult men and $<$1.4 mg/dl in adult women [319]. Although serum creatinine levels are a fairly good indicator of kidney function, multiple factors including age, sex, and ethnicity [320] affect its concentration so the use of a single set cutpoint may not be an appropriate way of defining adverse serum creatinine levels.

Creatinine can be measured via serum or urine. Serum creatinine exhibits significant individual differences [321]; while urinary creatinine and creatinine clearance show fewer individual differences and may provide a more reliable means of determining kidney function. Equations using serum creatinine to predict creatinine clearance include additional factors (e.g., age and body weight) in their prediction [322]. Reduced glomerular filtration rate (GFR), measured from serum creatinine, is associated with increased risk of cardiovascular disease and death [323]. Studies have shown that creatinine clearance predicts stroke and cardiovascular mortality [324].

Cystatin C is a cysteine protease inhibitor that is filtered out of the blood by the kidneys. As another marker of GFR, serum cystatin C is a measure of normal kidney function. Compared to serum creatinine levels (the primary clinical tool used for measuring renal function), cystatin C levels are independent of age, sex, and lean muscle mass. Hence, this is a promising biomarker for population studies. Additionally, multiple studies have indicated that cystatin C may be a more sensitive marker of kidney function than serum creatinine [131]. Cystatin C predicts all-cause and cardiovascular mortality [129, 325, 326], risk of cardiovascular disease [327], MI [328], stroke [328], and chronic kidney disease [329]. The correlation between high risk creatinine clearance and high risk cystatin C among people 65 years of age and over in NHANES III is 0.34 (Table 7a).

TABLE 7
PHI COEFFICIENTS AMONG HIGH RISK LEVELS OF MARKERS OF ORGAN FAILURE

(a) Ages 65+ in the NHANES III (N=2,741)

	Creatinine clearance	Peak flow	Cystatin C
Creatinine clearance		0.07**	0.34***
Peak flow			0.06**
Cystatin C			

(b) Ages 70–79 in the MacArthur Study of Successful Aging (N=654)

	Creatinine clearance	Peak flow
Creatinine clearance		0.11*
Peak flow		

$^{***}p < 0.0001$, ** $p < 0.001$, * $p < 0.01$, $^{+}p < 0.05$.

The *peak flow rate* provides an indicator of the functioning of the respiratory system. Peak expiratory flow (PEF) monitoring has been used as an objective measure of airflow obstruction. The normal range of PEF is 500–700 liter/min for men and 380–500 liter/min for women [330] but what is regarded as normal varies with differences in height and weight [331]. Studies have shown that PEF is related to mortality [332] and physical and cognitive functioning [333]. The correlation between peak flow and creatinine clearance in the MacArthur study is moderate (0.11, Table 7b).

An *electrocardiogram* (EKG or ECG) measures electrical impulses in the heart [134–136] and records as a graphic produced by an electrocardiograph. EKG results provide important diagnostic information on cardiac arrhythmias [334], MI [335], electrolyte disturbances [336], and ischemic heart disease [334]. A standard 12-lead resting EKG is often coded using Minnesota coding criteria [336]. The results are used to indicate probable and possible MI, and probable and possible left ventricular hypertrophy (LVH). A study based on national data showed that the age-adjusted prevalence rate of EKG-defined MI was 6.7% for those ages 40 and over which is somewhat higher than the prevalence of self-reported MI (5.8%). The prevalence was more than four times higher among those ages 65 and over compared to ages 40–64 [337].

4.8. MARKERS OF OXIDATIVE STRESS AND ANTIOXIDANTS

Oxidative stress and antioxidants are an example of a class of markers that seem to be theoretically important determinants of the aging process but are as yet not measured in such a way that they can be collected from large

populations. High levels of reactive oxidative species (ROS), enzymes important in cell signaling, have been shown to cause significant damage to cell structures. It has been suggested that ROS play an important role in the onset of age-associated loss in muscle mass (sarcopenia) [338], changes in the central nervous system, hearing loss [339], Parkinson's disease [340, 341], and AD [342–344]. In contrast, intrinsic [e.g., superoxide dismutase (SOD) and glutathione peroxidase] and extrinsic antioxidants (e.g., vitamins A, B, C, and E) affect aging and disease by combating oxidative stress [345]. Studies suggest that SOD may function as a tumor suppressor [346–348] while carotenoids may have preventive effects against both cardiovascular disease and cancer [349–352].

4.9. Genetic Markers

Genetic markers are another category of markers that are only beginning to be employed in population studies. The growth in ability to use these markers not only as additional independent indicators of risk but also as modifiers of risk for people with other behavioral, biological, or genetic characteristics will broaden the whole approach to including biomarkers in the analysis of population health outcomes. Only a small number of genetic indicators have been used broadly in population studies to date, and the results for many of the indicators have not been as clear-cut as expected given the animal literature, which led to their selection as candidates for genes influencing human health and longevity [353]. While we cannot review all of these markers, we will highlight promising results; this is just a brief mention of the genetic markers that are likely to be commonly determined in population surveys within the next decade.

The most commonly examined genetic indicator, and the one with the most evidence of a link to health outcomes, is *apolipoprotein E* (APOE), which has been used in analysis of a variety of health outcomes in many populations. There are three alleles of the APOE gene: e2, e3, and e4. Studies have shown high risks for late-onset AD among those with the APOE4 gene [354–359]. The APOE4 gene is also known to be associated with cardiovascular diseases such as heart attack, stroke, and coronary artery disease [360, 361].

Polymorphisms for the gene coding for *angiotensin-converting enzyme* (ACE) have also been examined in a number of population surveys. Polymorphisms in ACE have been shown to be relatively strongly related to circulating ACE and may be involved in cardiovascular and renal diseases [362], AD [363, 364], and human longevity [365, 366]; but not all investigations of the role of ACE have produced positive results [367, 368].

The number of candidate genes identified and investigated in large population surveys is likely to increase exponentially in a short time. For instance, the HTR2A genotype has also been associated with memory change and is likely to be included along with APOE as risk factors for cognitive loss [369]. A set of inflammatory polymorphisms related to IL-6 and CRP has been related to circulating levels of these markers, and while there are conflicting results as to how these relate to long-term health outcomes, they are likely to be increasingly included along with blood levels of these markers in future analyses [370, 371].

Mutations in mitochondrial DNA (mtDNA) accumulate with age and are among the genetic factors that may eventually be shown to be associated with longevity [353, 372]. A study of Italian populations indicated that mtDNA inherited variability may be involved in longevity and healthy aging [373]. Another Italian study found a specific link between longevity and the C150T mutation in leukocytic mtDNA [374]. Additionally, a Japanese study found that three mtDNA mutations were more prevalent among centenarians compared to noncentenarian controls [375].

Telomere length is another genetic indicator that is currently under investigation as either an indicator of the risk of aging or as a biological marker of the aging process *per se*. Although findings have consistently related decreased telomere length to increased age [376], investigations of the link between telomere length and remaining longevity have not produced consistent results [377, 378].

Identifying biomarkers for cancer is a rapidly growing scientific undertaking partly being fueled by genomic developments. Markers of DNA damage and repair provide hope for identification of markers that are related to risk for a wide variety of cancers [379, 380]. Work in other areas shows promise that serum autoantibodies that indicate chronic inflammatory, pro-oxidant conditions can serve as bioindicators of the risk of cancer development [381, 382].

5. Biomarkers and Mortality

The link between high risk levels of each biomarker and mortality indicates the relative potential of each marker individually to explain the likelihood of dying in older populations and to provide evidence of how this association varies across markers. Logistic regressions were used to estimate these relationships in the MacArthur study and hazard models in the NHANES analysis. The two cohorts are persons over age 40 from the NHANES III sample and the cohort ages 70–79 from the MacArthur Study of Successful Aging. Deaths in the MacArthur sample occurred in the 7.5 years after

TABLE 8
LINK BETWEEN PRESENCE OF RISK LEVELS OF INDIVIDUAL BIOMARKERS AND SUBSEQUENT MORTALITY[a]

	MacArthur: Age 70–79 7.5 years mortality (N=657)	NHANES III: Age 40+ Mortality from interview to 2000 (N=7,417)
	Odds ratios for mortality	
Systolic blood pressure	1.37	1.16*
Diastolic blood pressure	1.40	1.01
Pulse rate at 60 s	–	1.26*
Total cholesterol (total cholesterol/HDL in MacArthur)	0.87	0.98
HDL cholesterol	1.31	1.06
Glycosylated hemoglobin	1.34	1.31*
Body mass index (waist/hip ratio in MacArthur)	1.27	0.90
C-reactive protein	1.67*	1.00
IL-6	1.41	–
Fibrinogen	1.28	1.29*
Albumin	0.86	1.07
Cortisol	1.14	–
DHEA-S	1.39	–
Norepinephrine	1.49	–
Epinephrine	1.38*	–
Creatinine clearance	2.22	1.31*
Peak flow	2.18*	1.40*

Source: MacArthur, Seeman *et al.*, 2004 [163], calculated using logistic models. NHANES calculated from data using hazard models.

HDL = high-density lipoprotein; LDL = low-density lipoprotein; IL-6 = interleukin-6; DHEA-S = dehydroepiandrosterone sulfate.

*$p < 0.01$.

[a] Age, gender, and education controlled.

interview and up to 12 years after interview in the NHANES group. Odds ratios resulting from these regressions are shown in Table 8. The odds ratios indicate the relative likelihood associated with dying in years subsequent to the two surveys for each high risk biomarker. When the odds ratio is greater than 1, the likelihood of dying for those with the risk factor is higher than for those without the risk factor; when it is less than 1, the relative likelihood is lower for those with the risk factor.

A number of high risk levels of the biomarkers including SBP, pulse, HbA1c, fibrinogen, creatinine clearance, and peak flow are significantly related to mortality in the NHANES sample of middle-aged and older adults. The largest odds ratios are from biomarkers indicating organ functioning

such as creatinine clearance (OR=1.31) and peak flow (OR=1.40). In the MacArthur sample, which includes only older people, the only indicators linked to mortality were high risk peak flow (OR=2.18), CRP (OR=1.67), and epinephrine (OR=1.38).

It is hard to compare the results of the two samples given that they differ in age, location, and different statistical models in the equations; however, the results suggest the potential of the importance of some biomarkers that are not currently used as clinical indicators such as epinephrine and markers of inflammation. This has also been true in an analysis of the links between multiple biomarkers and mortality in Taiwan [383]. In addition, the results suggest that the importance of individual biomarkers in predicting health outcomes may be related to age, with many biomarkers potentially more important in predicting mortality at younger ages.

6. Interrelationships Among Biomarkers and Summary Measures of Biological Risk

In earlier sections, we showed the interrelationships among variables in each category. We now indicate the interrelationships among the cardiovascular, metabolic, inflammatory, HPA activity and SNS activity, and organ failure indicators for both the NHANES and the MacArthur samples in Table 9. If dysregulation in one marker or system is associated with dysregulation in multiple systems, the matrix should indicate high correlations. However, there are only a few moderate relationships among the high risk levels of these biomarkers in both samples. In the NHANES sample, the highest relationships are between CRP and BMI and leptin indicating the interaction of metabolic processes and inflammatory processes; strong correlations between creatinine clearance and cystatin C and a number of markers indicate the link between kidney functioning and a number of other processes. In the MacArthur sample, some of the strongest relationships are also between the markers of inflammation and the metabolic indicators.

Development of summary measures that incorporate multiple biological risk factors has been pursued in order to more effectively combine the information from multiple markers and also because of the observation that ''many individuals are exposed to several risk factors and small increases in multiple risk factors can lead to a substantial increase in overall risk, even if no single factor exceeds its clinically accepted threshold'' [7] (p. 95). Some of these summary measures focus on only a few physiological systems and others include more systems; some measures are more closely linked to

TABLE 9

PHI COEFFICIENTS INDICATING RELATIONSHIPS AMONG HIGH RISK LEVELS OF BIOMARKERS

(a) Ages 65+ in the NHANES 1999–2002 (NHANES III for leptin, creatinine clearance, peak flow, and cystatin C)

	TC	HDL	LDL[a]	TG[a]	GL	HbA1c	BMI	LEP[a,b]	CRP	FG	AL	CrCl[b]	PF[b]	CysC[b]
DBP	0.02	0.03	0.04	−0.04	−0.03	−0.05+	0.04	−0.02	0.02	0.01	−0.01	0.02	−0.04+	0.00
SBP	0.09***	−0.03	0.01	0.00	0.01	−0.02	−0.02	−0.02	0.06*	−0.00	−0.02	0.08***	0.07***	0.06*
PP	0.03	−0.01	−0.03	0.08+	0.09*	0.07*	−0.04	0.04	0.03	0.01	0.00	0.02	0.10***	0.00
PR	−0.00	0.00	−0.03	0.01	0.07+	0.10***	0.05+	0.01	0.07*	0.09***	0.05+	0.03	0.03	0.03
HC	0.01	−0.01	−0.04	−0.01	−0.05	−0.03	0.02	−0.04	0.03	0.12***	0.13***	0.20***	0.06+	0.25***
TC									0.04	0.06+	−0.05+	0.02	0.04+	0.01
HDL									0.01	0.04	0.02	−0.00	−0.04+	0.12***
LDL[a]									0.01	0.10*	−0.03	0.03	−0.06	−0.02
TG[a]									0.11***	0.03	−0.00	0.01	0.02	0.03
GL[a]									−0.01	0.02	0.03	−0.04	−0.01	0.05
HbA1c									0.05+	0.08***	0.05	0.02	0.04+	0.10***
BMI									0.16***	0.11***	0.09***	−0.11***	0.03	0.04+
LEP[a,b]									0.23***	0.08*	0.10**	−0.05	0.07+	0.11**
CRP												0.03	0.07**	0.13***
FG												0.04+	0.04+	0.12***
AL												0.08***	0.07**	0.05*

(b) Ages 70–79 in the MacArthur Study of Successful Aging (N=654, N=363 for homocysteine)

	Tot/HDL	HDL	GHb	BMI	Wasit/Hip	CRP	IL6	FG	AL	COR	DHEAS	NE	EPI	CrCl	PF
DBP	−0.00	0.07	−0.00	0.08+	0.06	−0.01	0.09+	−0.02	−0.03	0.01	0.02	0.06	−0.03	−0.02	−0.01
SBP	0.05	−0.01	0.03	0.09+	0.09	0.06	0.11*	0.04	0.00	−0.01	−0.09+	0.04	0.01	0.06	0.01
HC	0.06	0.17*	−0.01	0.00	0.09	0.05	0.15*	0.02	0.06	−0.14*	−0.06	−0.05	−0.09	0.01	0.04
Tot/HDL						0.09+	0.03	0.18***	−0.15**	−0.03	−0.06	−0.06	−0.07	−0.00	−0.07
HDL						0.09+	0.03	0.10*	−0.02	−0.07	−0.03	−0.07	−0.10+	−0.07	−0.15**
GHb						0.17***	0.10+	0.10*	−0.02	−0.13**	−0.02	−0.10+	−0.05	−0.07	−0.02
BMI						0.13**	0.09+	0.09+	0.01	−0.03	−0.01	−0.01	−0.07	−0.01	−0.00
Waist/Hip						0.06	0.07	0.02	−0.05	−0.02	−0.07	−0.06	−0.12*	−0.11*	−0.06
CRP										0.01	−0.03	0.02	−0.01	0.02	0.13**

IL-6	−0.04	0.02	0.07	−0.03	0.00	0.06
FG	−0.07	−0.08	−0.06	−0.00	−0.06	0.04
AL	−0.04	0.07	−0.03	0.02	0.02	0.07
COR					0.09+	−0.00
DHEA-S					0.02	0.05
NE					0.06	0.13**
EPI					0.27***	0.10+

Ns for analysis: NHANES 1999–2002 nonfasting biomarkers (N=1,884), fasting biomarkers (N=938); NHANES III nonfasting biomarkers (N=2,741), fasting biomarkers (N=1,172); NHANES III homocysteine nonfasting biomarkers (N=1,407), fasting biomarkers (N=571).

*** p <0.0001, ** p <0.001, * p <0.01, + p <0.05.

DBP=diastolic blood pressure; SBP=systolic blood pressure; PP=pulse pressure; PR=resting pulse rate; HC=homocysteine; TC=total cholesterol; Tot/HDL=total cholesterol/HDL; HDL=high density lipoprotein; LDL=low-density lipoprotein; TG=Triglycerides; GL=blood glucose; HbA1c=glycosylated hemoglobin; BMI=body mass index; LEP=leptin; waist/hip=wasit-to-hip ratio; CRP=C-reactive protein; IL-6=interleukin-6; FG=fibrinogen; AL=albumin; COR=cortisol; DHEA-S=dehydroepiandrosterone sulfate; NE=norepinephrine; EPI=epinephrine; CrCl=creatinine clearance; PF=peak flow; CysC=cystatin C.

[a] Fasting biomarkers: LDL, triglycerides, blood glucose, leptin.

[b] Correlations based on biomarkers from NHANES III.

specific health outcomes like cardiovascular disease while others propose to explain a variety of health outcomes.

The *Framingham risk score* is a widely used index of risk for CHD [33, 384–389]. The Framingham score assigns points to different major cardiovascular risk factors including blood pressure, total cholesterol, LDL cholesterol, HDL cholesterol, and fasting blood glucose. It also includes risk related to age, gender, and smoking. For those without cardiovascular disease, the probability of CHD onset within a certain period of time is estimated. The Framingham risk score is widely used in clinical settings based on its proven ability to predict cardiovascular disease and CHD especially for women [390]. The Framingham risk score has been also shown to predict absolute risk accurately for populations other than those in North America [391–394] although some recent studies have questioned its validity in other settings [390].

Metabolic syndrome is a group of major risk factors that characterize an insulin resistance syndrome or Syndrome X [33] (p. 2488), which has been related to increased risk for cardiovascular disease and mortality [395–397]. The metabolic syndrome score is a count (0–5) of the number of the following abnormalities: hypertension, glucose dysregulation, hypertriglyceridemia, low HDL, and central obesity—based on clinical cut points [33, 398, 399]. A person with three or more of these five abnormalities is considered to have metabolic syndrome.

Allostatic load is a summary measure that is based on theories about aging and the cumulative physiological responses to stressors [400]. This summary measure involves multiple systems that are part of the body's stress response but that may become dysregulated with chronic physical or mental stress and old age. Initially, allostatic load was measured in the MacArthur study based on 10 biological markers that represent physiological activity across the cardiovascular system, the metabolic system, the HPA and the SNS including SBP, DBP, WHR, ratio of total/HDL cholesterol, HDL cholesterol, HbA1c, cortisol, norepinephrine, epinephrine, and DHEA-S [102]. Allostatic load was measured as the number of markers out of 10 for which the subject scored in the upper 25% of the distribution. This measure has been shown to predict mortality and decline in physical and cognitive functioning [102, 309].

Subsequent analyses have included additional markers that represent renal functioning, lung capacity, inflammation and coagulation, and addition of these markers increased the explanatory power of the measure [160]. These analyses have also shown that allostatic load is a better predictor of health outcomes in the older MacArthur sample than the set of individual markers or the indices of the cardiovascular and metabolic markers [309].

Seeman and colleagues have explored several alternative approaches to measurement including allowing differential contribution of individual

indicators through their entire range of values and different weights for different outcomes [160, 401, 402]. While these refined approaches indicate that differential weighting of the individual components of biological risk by the outcome of interest might be optimal, the original count index and the more complex approach do not differ significantly in their predictive ability [7].

7. Surveys with Biomarkers

Biomarkers are available in many large samples representative of national populations and communities, which allow examination of the diversity of biomarkers within the population and the large numbers needed for examination of longitudinal change. They are designed to examine the relationship of not only the risk associated with biological factors but also social and economic factors and the interaction among these risks. They do not expect to provide evidence of new biological relationships or risks for health outcomes but they could be used to identify important interactions between biological factors and health outcomes. These surveys generally include measurement of risk factors and physiological states known to be related to highly prevalent major health outcomes. We describe a selection of these studies below. In each case, we give some idea of the biomarkers available but in many cases we are not exhaustive in our listing. Also, because many of the studies have stored samples, biomarkers are added regularly from new assays.

Our analyses have used biomarker information from the NHANES, which include interviews, clinical exams, and extensive laboratory analysis which results in the most extensive set of biomarkers for a large population. These studies are undertaken by the National Center for Health Statistics, and exams and biological specimens are collected by medical staff working in mobile exam units in trucks that move across the country. NHANES, with the exception of the first study, is cross-sectional except for passive follow-up of administrative death records and Medicare records. The available biomarkers are too extensive to be mentioned individually but in addition to those mentioned above other indicators include hematology antibody tests, hormones, toxicology, and assessments of anemia and sexually transmitted diseases (STDs). Exams include vision, audiometry, periodontal assessments, cardiovascular fitness, physical functioning, balance, cognition, and reaction.

The MacArthur Study of Successful Aging was the first large-scale study to collect information on a significant number of biomarkers in the home rather than in a medical setting [403]. This survey was of people aged 70–79 in three

communities and biomarkers were collected at multiple time points. A phlebotomist collected blood samples and interviewers collected overnight urine collections. Many of the measures available from this study have been indicated above. Some were from assays done at the time of collection and others from stored samples (e.g., antioxidants, homocysteine, folic acid, CRP, fibrinogen, IL-6, and extraction of DNA). There are additional performance tests for balance, walking ability, strength, and cognitive functioning.

The number of large population and community studies including the collection of biomarker data has multiplied in recent years partly in response to the technological changes that have allowed interviewers or respondents rather than medical professionals to collect samples. These developments include the use of dried blood spots [404], and buccal swabs and salivary assays for DNA. The Health and Retirement Survey is a nationally representative longitudinal study of the US population over age 50. It has been ongoing since 1992 and added the collection of biomarkers and performance measures in 2006 [405]. This study collects blood samples using the dried blood spot, which have been assayed for HbA1c, total cholesterol, HDL cholesterol, and CRP. DNA has been extracted from saliva. Participants also completed several performance tests for strength, balance, and lung function.

National samples from other countries have also introduced these approaches to collecting information on biomarkers. The Taiwan Biomarker Project has collected a set of biomarkers [406], as has the English Longitudinal Study of Aging [407]. The Mexican Family Life Survey and the Indonesian Family Life Survey are both collecting blood using dried blood spots [408]. Additionally, the Mexican Family Life Survey is collecting information on anemia at the time of the survey using a hemocue meter.

8. Future of Biomarkers in Studying Aging Populations

The increase in the number of population and community studies including the collection of biomarker data has resulted from theoretical imperatives, scientific advances, and improvements in collection opportunities. The theoretical demands require a fuller explanation of how the aging process proceeds. The scientific advances have dramatically increased our knowledge of the multiple biological pathways affecting the aging process. The collection opportunities have increased with the development of less invasive measurement offered by salivary and dried blood spot assays. The future is likely to see further expansion of biomarker collection using saliva not only for DNA but also for RNA and certainly an increase in markers based on scanning. Many samples for well-characterized populations are available now for further genetic analysis; development of inexpensive genotyping techniques

will result in an expansion of genetic biomarkers. The developments in metabonomics, analysis of metabolic profiles, and proteomics will lead to the inclusion of many new classes of biomarkers.

Multiple biomarker measurements that are more indicative of the physiological response to challenge are likely to be included in population surveys in the future. Monitoring through telephone or small electronic device (e.g., paging devices, palmtop computers, and programmable wristwatches) will be increasingly used to collect and stimulate responses.

Finally, further methodological developments will be required to analytically integrate the increasingly complex indicators that will be collected. The number of biomarker indicators and the interrelationships among them demand new analytic approaches.

Acknowledgement

This work was partially supported in the U.S. by the National Institute on Aging Grants P30 AG17265 and T32AG0037.

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VASCULAR CALCIFICATION INHIBITORS IN RELATION TO CARDIOVASCULAR DISEASE WITH SPECIAL EMPHASIS ON FETUIN-A IN CHRONIC KIDNEY DISEASE

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0065-2423/08 $35.00
DOI: 10.1016/S0065-2423(08)00406-X

1. Abstract

The mortality rate is extremely high in chronic kidney disease (CKD), primarily due to the high prevalence of cardiovascular disease (CVD) in this patient group. Apart from traditional Framingham risk factors, evidences suggest that nontraditional risk factors, such as inflammation, oxidative stress, endothelial dysfunction, and vascular calcification also contribute to this extremely high risk of CVD. Disturbance in the mineral metabolism, especially in the ions of Ca and PO_4, are linked to enhanced calcification of blood vessels. Although the mechanism(s) of this enhanced calcification process are not fully understood, current knowledge suggests that a large number (and an imbalance between them) of circulating promoters and inhibitors of the calcification process, that is, fetuin-A (or α2-Heremans–Schmid glycoprotein, AHSG), matrix-Gla protein (MGP), osteoprotegerin (OPG), osteopontin (OPN), bone morphogenetic proteins (BMPs), and inorganic pyrophosphate (PPi), are involved in the deterioration of vascular tissue. Thus, an imbalance in these factors may contribute to the high prevalence of vascular complications in CKD patients. Among these mediators, studies on fetuin-A deserve further attention as clinical studies consistently show that fetuin-A deficiency is associated with vascular calcification, all-cause and cardiovascular mortality in CKD patients. Both chronic inflammation and the uremic milieu *per se* may contribute to fetuin-A depletion, as well as specific mutations in the *AHSG* gene. Recent experimental and clinical studies also suggest an intriguing link between fetuin-A, insulin resistance, and the metabolic syndrome.

2. Introduction

Premature atherosclerotic CVD is a leading cause of morbidity and mortality in patients with CKD [1]. The annual mortality rate due to CVD is approximately 10- to 20-fold higher in CKD patients than in the general population [1, 2]. Many factors contribute to this high risk. Although traditional Framingham risk factors, such as hypertension, dyslipidemia, and diabetes mellitus (DM), may account for a large proportion of the excessive burden of CVD in this patient population, they do not fully explain it. Recent studies suggest that nontraditional risk factors, such as inflammation, oxidative stress, and vascular calcification tendency, may also contribute [2]. CKD patients are often present with vascular calcification and atherosclerosis-related calcification of the intima appears to be of particular clinical

importance, although the cardiac valves and medial arterial layer are also often involved [3]. Although vascular calcification in the vessel wall is uncommon in younger age groups in the general population, it is extremely common among CKD patients [4–7]. In one study it was even noted in young patients, 20- to 30-year old [6]. The prevalence of vascular calcification among dialysis patients is very high and increases as dialysis vintage increases [4]. Moreover, the extent of vascular calcification and arterial stiffness are strong predictors of CVD and all-cause mortality in the dialysis population [8–10].

The mechanism(s) of vascular calcification is still not well defined. It is generally thought that under certain pathological conditions ectopic calcification develops as a passive degenerative process, leading to uncontrolled precipitation of calcium (Ca) phosphate (PO_4) that is associated with tissue necrosis and/or metabolic Ca and PO_4 imbalance. Generally, multiple hormones acting systematically and cytokines acting locally regulate bone remodeling and that bone may exert an endocrine regulation that is determined by these molecules. Indeed, a recent report by Lee *et al.* [11] demonstrated that osteoblasts regulate glucose homeostasis and energy metabolism, suggesting that bone may contribute to the development of metabolic disorders. Thus, this indicates that bone has additional functions that change by abnormal pathological conditions. Notably, recent reports from clinical and experimental studies document that vascular calcification is an active, rather than passive, and a biologically regulated process that shares the characteristics of bone formation and bone repair [12–16]. Moreover, this process is associated with an imbalance of a large number of promoters and inhibitors of the calcification process [17–19]. Thus, vascular calcification may result due to imbalance of the equilibrium between promoters and inhibitors, and it is increasingly evident that the concentration of Ca and PO_4 is not the only factor influencing ectopic calcification [20]. There are many promoters and inhibitors involved in the calcification process and most of them are closely associated with comorbidity and mortality in CKD patients, such as Ca, PO_4, vitamin D, parathyroid hormone (PTH), and markers of inflammation and wasting, such as C-reactive protein (CRP), cytokines, fibrinogen, and serum albumin. Recently, a number of bone-associated proteins, including fetuin-A (or AHSG), MGP, OPG, OPN, BMPs, and PPi, have been suggested to play a pivotal role in the prevention of the calcification of soft tissues under physiological conditions [21–24]. In this chapter, we will discuss protein inhibitors of the vascular/ossification process. Although our focus will be primarily on fetuin-A, we will also discuss the role of other common vascular calcification inhibitors, such as MGP, OPG, OPN, BMP-7, and PPi.

3. Extraosseous Calcification

The calcification or mineralization process occurs as a normal physiological process in bones and teeth. Bone formation is classified into two types: endochondral bone formation that involves chondrogenesis and a cartilage intermediate, and intramembranous bone formation that derives from direct differentiation of mesenchymal stem cells into bone-forming osteoblasts. Certain pathological conditions can promote the mineralization process in different tissues and organs (ectopic or extraosseous calcification), such as vessels, heart, soft tissues, the periarticular region, skin, and other solid organs. It is plausible that the restriction of mineralization to bones and teeth occurs due to presence of active inhibitors that prohibit calcification in soft tissues. Pathological calcification has many similarities to physiological calcification [12, 16] and is classified as metastatic or dystrophic calcification [25]. In metastatic calcifications, there is a disturbance of Ca and/or PO_4 metabolism and this may occur in the heart and blood vessels as well as in other soft tissues. Dystrophic calcifications are, on the other hand, seen in human tissues in the absence of known Ca-PO_4 imbalances; for example, in atherosclerotic plaques or necrotic tissues [25, 26].

Based on the histological features, vascular classification is classified into four types [27], which can be present alone or in combinations and each is related to a characteristic type of vascular disease processes. **(A)** Cardiac valve calcification occurs in response to mechanical stress and inflammatory stimuli, which recruit dystrophic mineralization and nonenchondral ossification processes to deposit of Ca. Valvular calcification is likely to be initiated through mechanisms distinct from atherosclerotic calcification [28–31]. **(B)** Intimal atherosclerotic plaque calcification is a type of dystrophic calcification, which involves endochondral ossification initially characterized by necrotic cells and inflammation as well as lipoprotein and phospholipid complexes [12, 32]. **(C)** Medial vascular calcification is the nonendochondral ossification process of the arterial tunica media, which is more similar to intramembranous bone formation. The media calcification (also referred to as Mönckeberg's sclerosis) is primarily associated with aging, DM, and CKD and is an end result of the inflammation and calcification of the atherosclerotic plaques often initiated early during disease progress [33]. In medial artery calcification, the mineralization is similar to intramembranous bone formation and odontogenesis in which no cartilaginous precursor is required, and BMP-2 and homeoprotein Msx2-dependent signaling is a central feature of the mineralization process [34–36]. Mineralization occurs initially at matrix vesicles associated with extracellular matrix fibrils [37]. Although the aortic expression of Msx2 and Msx1 is upregulated in diabetic patients [34, 36], this has not yet been studied in the setting of CKD. The medial

calcification may be present in combination with calcification in intima and both are common in CKD as well as in diabetic patients [38]. It has been argued by London and Drueke [39] that the clinical distinction between the two coexisting conditions associated with arterial disease, arteriosclerosis, and atherosclerosis may not be easy to define. Whereas arteriosclerosis (medial calcification) is primarily a medial degenerative condition associated with aging that leads to the stiffening of the elastic layer of the arterial wall and does not obstruct the arterial lumen, atherosclerosis (intimal calcification) is a disease process that typically results in narrowing or occlusion of arteries [39]. The decrease in arterial wall elasticity in arteriosclerosis condition may explain the findings that medial calcification has emerged as the more significant predictor of lower extremity amputation and cardiovascular mortality risk in type-2 DM [8, 38]. London *et al.* [3] showed that dialysis patients with intimal calcifications were older and characterized by a history of traditional risk factors, while patients with medial calcifications were younger and characterized by a longer duration of dialysis treatment and derangements in their $Ca \times PO_4$ balance. Eventually, both contribute to the increased risk of mortality in CKD patients [40, 41]. **(D)** Vascular calciphylaxis is the fourth component of the widespread soft tissue calcification that occurs when the physiological $Ca \times PO_4$ solubility threshold is exceeded [42]. It is observed occasionally in uremia (also called calcific uremic arteriolopathy) and is characterized by ischemic ulceration of the skin due to metastatic calcification of subcutaneous tissue and small arteries.

4. Vascular Calcification: A Tsunami in CKD Patients

Although vascular calcification in CKD is a feature recognized since the nineteenth century it has been more or less ignored due to poor understanding of its impact on patient outcome until recently. The interest in vascular calcification has increased in the last decade when recent studies showed that a high incidence of vascular calcification is associated with poor clinical outcome [6, 9, 43–46]. In CKD, calcification of blood vessels occurs in the media in the case of Mönckeberg's medial sclerosis and calcific uremic arteriolopathy and/or in the intima, in the case of atherosclerosis, where calcification is scattered and has an irregular pattern associated with atherosclerotic occlusive lesions. This process is seen in the aorta and coronary arteries and other muscular arteries and starts already during childhood and adolescence CKD patients [33, 40]. Although medial calcification is common with aging it is also a common feature of the vascular disease seen in uremia and DM [33, 40, 47]. It primarily affects the aorta and extends to the peripheral smaller vessels [16, 33, 40]. In addition to its prevalence in adult

CKD patients, medial arterial calcification is thought to underlie the unexpectedly high coronary Ca scores and decreased arterial elasticity found in pediatric dialysis patients [48]. It is plausible that both types of calcifications coexist in most of the patients [49] and contribute to cardiovascular events most likely through different mechanisms in CKD patients. Therefore, the combined effect of both processes would probably explain the extremely high rates of cardiovascular and all-cause mortality in this specific patient population [50, 51]. Indeed, in coronary arteries, the presence of vascular calcification is highly correlated with the risk of myocardial infarction, and sudden death [52–54].

The prevalence and extent of vascular calcification is a strong predictor of CVD and all-cause death in hemodialysis (HD) [9] and peritoneal dialysis (PD) [10] patients. Of note, vascular calcifications in CKD patients differ from that in the general population not only in the type and localizations of the calcifications but also because it is common in childhood/adolescence age and a central characteristic of the progressive atherosclerosis observed among CKD patients [6, 7]. Together with the impact of several risk factors, this fact will have a great impact on the rate, extension, and severity of the vascular calcifications as well as the impact on poor outcome [9].

5. Role of Calcification Inhibitors

The concentrations of Ca and PO_4 are physiologically balanced at levels within reach of the solubility product. Pathologically, when the serum $Ca \times PO_4$ product increases with accumulation of PTH fragments (which agitate normal Ca-PO_4 homeostasis), widespread tissue deposition of amorphous Ca-PO_4 may occur. For many years, this process of vascular and soft tissue calcifications was considered solely as a result of a passive deposition of hydroxyapatite crystals due to an elevated Ca-PO_4 ion product. However, mounting evidence has shown that this is a complex and highly regulated process that involves inhibitors, inducers, and cell differentiation processes [55]. Moreover, this process is governed by factors that closely resemble Ca deposition in bone tissue, which (particularly in the uremic milieu) is due to vascular smooth muscle cells (VSMCs) damage as well as disturbance of circulating and cellular inhibitors of Ca and PO_4 precipitation. This has led to the concept that multiple mechanisms, including loss of inhibition, induction of bone formation, circulating nucleational complexes, and cell death may induce this specific pathology [56]. In fact, the vascular calcification process requires changes in the phenotype of VSMCs and the expression of several proteins normally involved in bone metabolism. Molecular genetic techniques and *in vitro* models have repeatedly revealed the necessity of

inhibitory mechanisms to prevent ectopic calcification. A growing number of such molecules have been identified using mutational analyses, and animal knockout models have confirmed the role of a number of proteins in regulating vascular calcification. Among them, fetuin-A, MGP, OPG, OPN, BMP-7, and PPi have attracted interest and have been identified as important natural inhibitors of vascular calcification.

6. Fetuin-A

6.1. Chemistry and Metabolism

Fetuin-A, also known as α2-Heremans–Schmid glycoprotein (AHSG), is a major circulating glycoprotein of the cystatin superfamily of cysteine protease inhibitors, also including histidine-rich glycoproteins and kininogens. The fetuin family constitutes a set of orthologous plasma proteins found in human, sheep, pig, cow, and rodents. Human fetuin-A was discovered by Heremans [57] and Schmid [58] and renamed as fetuin-A after the discovery of fetuin-B. Fetuin-A is produced by multiple tissues during fetal development, whereas in the adult it is synthesized by hepatocytes, secreted into blood and accumulates in the skeleton during mineralization, due to its high affinity for hydroxyapatite [59]. Despite its deposition as a noncollagenous protein in mineralized bones and teeth, it has not been possible to demonstrate the synthesis of fetuin in calcified tissues; it is likely that the fetuin is transported there via the serum. Fetuin-A is abundant in extracellular fluids with serum concentrations ranging from 0.4 to 1.0 g/liter and has a molecular mass of approximately 60 kDa [60].

The *AHSG* gene in humans is located on chromosome 3q27 and consists of seven exons and six introns [61]. The gene consists of a tandem arrangement of two cystatin-like domains and a unique third C-terminal domain, rich in proline and glycine and not present in other mammalian cystatins. Each domain is approximately 120 amino acids long. The fetuin-A protein molecule consists of two polypeptide chains produced through posttranslational cleavage from a single polypeptide by enzymatic proteolysis [62] and held together by a single interchain disulfide bridge. Posttranslational and secondary modifications, such as phosphorylation, *N*- and *O*-glycosylation, are common for the fetuins from different species. It has been calculated that the average phosphorylation degree of the circulating human fetuin-A in serum is approximately 0.2 mol/mol of the protein [63]. The three N-linked and three O-linked chains of oligosaccharide contain various sugar molecules and the terminal sugar residues are rich in sialic acid, which contributes to the net negative charge of the molecule [64].

The fetuin-B protein is a serum glycoprotein with overlapping tissue distribution with fetuin-A, but its level in plasma (5 mg/liter) is just a fraction of that of fetuin-A in humans. The 382 amino acids long protein shows a 22% sequence similarity with fetuin-A and a 61% amino acid identity is shared between human, mouse, and rat proteins [65]. Like its fetuin-A counterpart, the fetuin-B mRNA level is downregulated in the liver following an induced inflammation in rat [65]. Functional analysis revealed that fetuin-B, similarly to fetuin-A, is an inhibitor, albeit less active, of basic calcium phosphate precipitation. In contrast with fetuin-A, the amount of fetuin-B protein in human serum is higher in females than in males [66].

6.2. Methods to Analyze Serum Fetuin-A

Commercial enzyme-linked immunosorbent assays (ELISA) are the predominant analysis technique for fetuin-A analysis in serum and the antibodies used are highly sensitive and specific for the protein with no cross-reactivity with sera of other animal species (mouse, rat, rabbit, sheep, goat, cattle, swine, and horse). "Sandwich" ELISA assays are available from, for example, Epitope Diagnostics, Inc. (San Diego, United States) and BioVendor Laboratory Medicine, Inc. (Modrice, Czech Republic). The 96 wells plates are coated with polyclonal anti-human fetuin-A-specific antibody. The fetuin-A which is present in the samples (10–100 μl needed) is captured by this antibody and unbound protein is washed away. Then a horseradish peroxidase (HRP) conjugated polyclonal anti-human fetuin-A antibody is added to the wells followed by a substrate solution. The absorbance at 450 nm is proportional to the fetuin-A concentration in the sample. The normal serum fetuin-A range is 0.4–1.0 g/liter. As another alternative for analysis, Nephelometry (e.g., Dade Behring BN II, Newark, DE) is a fully automated system for plasma protein determinations and combines reliability and high sensitivity with cost-effective rapid performances. Laboratories having access to such a machine have a good alternative for fetuin-A analysis. In the Dade Behring assay, serum samples are centrifuged and exposed to a polyclonal rabbit anti-human fetuin-A antibody identical to that used in ELISA methodology. The assay does not cross-react with fetuin-B and other serum proteins or with proteolytic fragments of fetuin-A. The assay range reported is from 0.05 to 3.5 g/liter (CV$<$10%) [67]. In order to minimize nonspecific turbidity, which is often the case in uremic samples, fresh serum is preferable than plasma for this analysis. Alternatively, lipemic sera should be cleared with a clarifying agent or a non-nephelometric assay, such as ELISA should be used.

6.3. Function of Fetuin-A

Fetuin-A is multifunctional molecule and it has been implicated in several diverse functions, including regulation of osteogenesis and bone resorption [68], prevention of ectopic vascular calcification by inhibiting formation of hydroxyapatite crystals [20, 69, 70], activation of hepatocyte-growth factor [71], response to systemic inflammation [72], and regulation of cytokine-dependent osteogenesis. Moreover, fetuin-A can act as a TGFβ antagonist, which is achieved by binding of this growth factor to a β-glycan-like domain of the fetuin-A molecule. Thus, via interaction with the insulin receptor tyrosine kinase activity fetuin-A seems to be involved in insulin resistance [72, 73].

6.4. Role of Fetuin-A in the Calcification Process

One of the major functions of fetuin-A is its ability to inhibit the hydroxyapatite formation by reducing crystal formation even in cell-free solutions containing Ca and PO_4 and consequently inhibit ectopic $Ca \times PO_4$ ion precipitation. This action of precipitation inhibition is mediated by calciprotein, which is transient formation of stable colloidal spheres, containing fetuin-A and basic Ca and PO_4 [70]. Calciprotein particles may serve to transport and remove insoluble Ca precipitates from extraosseous sites to bone tissue acting as a cleaner of calcified foci. Accordingly, it has been suggested that fetuin-A inhibits the *de novo* formation and precipitation of the apatite precursor mineral, basic Ca-PO_4, only transiently and does not dissolve basic Ca-PO_4 once it is formed [69, 70]. Therefore, fetuin-A can hinder undesirable calcification in tissues and circulation without inhibiting bone mineralization. Its function of inhibiting soft tissue calcification is achieved by forming a soluble colloidal microsphere of fetuin–Ca-PO_4 complex in the blood stream. Recently, it has been shown that fetuin-A circulates in the blood in a complex with MGP and Ca-PO_4 precipitates. Price *et al.* [74] discovered that in etindronate-treated rats fetuin-A exists as a high molecular mass complex of fetuin-A (80%), as a Ca-PO_4 mineral (18%), and as a minor part bound to MGP (2%). There is evidence that high-dose vitamin D treatment, leading to extensive calcification of the artery media, is associated with the presence of fetuin–mineral complex [75]. It has also been observed that the timing of vitamin D-induced artery calcification correlates with the timing of the maximal increase in serum fetuin–mineral complex levels and this complex was undetectable in the blood of rats in which artery calcification was inhibited by ibandronate (a bone resorption inhibitor), or OPG [75]. Although the mechanisms are presently unclear, it is plausible that this complex plays a role in the removal or clearance of mineral component

out of soft tissues, thus preventing and even repairing unwanted calcifications. So far, the high molecular mass fetuin-A-containing complex was found in rat sera and it is yet unclear whether it is identical to calciprotein particles and present in humans.

The relevance of fetuin-A and its inhibitory activity on hydroxyapatite formation has been documented in animal studies after specific depletion of fetuin-A from the serum. Schäfer *et al.* [20] demonstrated that fetuin-A-deficient mice develop widespread ectopic calcifications of different organic tissues, including kidney, heart, lung, skin, and vasculature. Notably, the severity of the developed calcifications seems to depend on the genetic background of the mice. They also observed that fetuin-A-deficient mice with the calcification-prone DBA/2 genetic background spontaneously develop severe and progressive ectopic calcifications [20]. This may subsequently lead to renal failure due to intratubular obstruction, and secondary hyperparathyroidism. On the other hand, fetuin-A-deficient mice with a C57Bl/6 genetic background (which is relatively more resistant to calcification) require additional stimuli, such as vitamin D treatment, to develop ectopic calcifications. Thus, mice lacking fetuin-A can develop soft tissue and intravascular calcification, especially when challenged with vitamin D [20]. *In vitro* data also support the importance of fetuin in the studies using primary osteoblast cultures, and salt precipitation assays have demonstrated that fetuin-A inhibits apatite formation [69]. Additionally, fetuin-A can act in VSMCs by inhibiting formation and intracellular calcification of matrix vesicles [76].

6.5. Role of Fetuin-A in Inflammation

There is a general agreement that fetuin-A is downregulated during inflammation and it has therefore considered as a negative acute phase reactant. Lebreton *et al.* [77] observed more than 25 years ago that during acute inflammation fetuin-A levels decreased. The anti-inflammatory property of fetuin-A was demonstrated by the role of fetuin-A to suppress TNF release from lipopolysaccharide (LPS)-stimulated macrophages *in vitro* [78] as well as *in vivo* in an LPS-independent model of acute inflammation [79]. Of note, it has been shown that interleukin-1β (IL-1β) downregulates the fetuin-A hepatic mRNA level [80]. In accordance, in LPS-challenged mice hepatic fetuin-A mRNA is markedly downregulated [81]. Hence, fetuin-A may have important functions in inflammation, such as limitation of cytokine production by macrophages [82] and protection against TNF [83]. Several reports have also suggested that fetuin-A may play a general role in phagocytosis regulation and innate immunity. Fetuin-A can act as an opsonin [84], it quenches the oxidative burst associated with the uptake of apatite crystals

by neutrophils [85], and it forms antibody complexes involved in marking and removal of apoptotic neutrophils [76]. Moreover, low fetuin-A serum concentrations have been found to be related with depressed cellular immunity [86] and nonspecific host defense [87] (Table 1)

Clinical studies suggest that low fetuin-A serum level is a predictor of poor outcome in patients with liver cirrhosis and liver cancer [88] as well as in CKD patients [89–92]. Although these reports do not necessarily infer causality, they are fully compatible with a role of fetuin-A in clearing insoluble remnants and link fetuin-A deficiency with inflammatory state. It is believed that fetuin-A has antifibrotic activity [72, 93], and can inhibit apoptosis of VSMCs [76]; a process known to contribute to the destabilization of atherosclerotic plaque. Recent evidence suggests that extraossoeus calcification is not only a passive degenerative process but also involves active inflammation [7, 91, 94]. Notably, also other inflamed patient groups, such as systemic lupus erythematosus patients, have an increased coronary calcification burden [95]. Hence, there may be several reasons why a state of chronic inflammation may promote vascular calcification and, in particular, mediators and inhibitors, such as leptin [96], MGP [97], TNFα [98], BMP [99], and OPG [100], may be related to a process of accelerated vascular calcification. Clinical data also suggest that the combination of inflammation and low fetuin-A may further aggravate the vascular calcification process.

6.6. Novel Link Between Fetuin-A and the Metabolic Syndrome

Fetuin-A inhibits insulin receptor autophosphorylation and subsequent downstream signaling *in vitro* [101, 102]. In accordance, injection with human recombinant AHSG inhibited insulin-stimulated tyrosine phosphorylation of the insulin receptor and insulin receptor substrate-1 in liver and skeletal muscle in rat [101]. In addition, fetuin-A-deficient mice display improved insulin sensitivity and are resistant to weight gain on a high-fat diet [73]. Furthermore, a link between fetuin-A and insulin resistance was suggested by *in vitro* studies demonstrating that fetuin-A inhibits (in a dose-dependent manner) the insulin-stimulated tyrosine kinase activity of the insulin receptor, insulin receptor autophosphorylation, and insulin substrate-1 phosphorylation [103]. These effects were corroborated *in vivo* in rat liver and skeletal muscle following acute injection of human recombinant AHSG [104] and in AHSG-null mice. These mice exhibit significantly enhanced insulin sensitivity and are resistant to weight gain on a high-fat diet [105]. In humans, serum AHSG levels have been reported to be associated with insulin resistance and fat accumulation in the liver [106], as well as with regulation of weight gain through modulation of adipocyte β2-adrenoceptor function [107, 108]. Also, in patients with coronary artery disease (CAD),

TABLE 1
MAJOR FINDINGS IN THE CLINICAL STUDIES ON SERUM FETUIN-A IN CHRONIC KIDNEY DISEASE PATIENTS

	Study	Study type	Number and category	Conclusions
1	Ketteler *et al.*, 2003 [89]	Cross-sectional; Prospective (32 months) *ex vivo*	312 HD pts	Serum fetuin-A concentration was lower in HD pts compared to healthy controls. Fetuin-A deficiency was associated with inflammation and a predictor of all-cause and CV mortality. Sera from pts on long-term dialysis with low fetuin-A concentrations showed impaired *ex vivo* capacity to inhibit $Ca \times PO_4$ precipitation. Reconstitution of sera with purified fetuin-A returned this impairment to normal.
2	Stenvinkel *et al.*, 2005 [90]	Cross-sectional; Prospective (3.5 years)	258 CKD stage 5 pts	Low fetuin-A is associated with wasting-inflammation, presence of carotid plaques, CV, and all-cause mortality. Patients with the AHSG 256Ser allele had lower fetuin-A levels and higher risk of all-cause and CV mortality.
3	Wang *et al.*, 2005 [91]	Cross-sectional; Prospective (mean 32 months)	238 PD pts	Low serum fetuin-A showed an association with wasting and inflammation. Every 0.01 g/liter increase in serum fetuin-A was associated with a 6% decrease in the risk of valvular calcification independent of CRP and $Ca \times PO_4$ product. Lower serum fetuin-A was associated with higher all-cause mortality and fatal and nonfatal CV events (significance lost when atherosclerotic vascular disease, valvular calcification, wasting, and inflammation were included in the model).
4	Mehrotra *et al.*, 2005 [123]	Cross-sectional	88 Type-2 DM pts (58 pts with DN)	Serum fetuin-A level was significantly higher among diabetic pts with DN compared to diabetics with no albuminuria. High fetuin-A levels were positively associated with CAC score.

5	Moe *et al.,* 2005 [122]	Cross-sectional	68 CKD stage 5 pts	Fetuin-A was negatively correlated with CAC scores and positively associated with OPG levels.
6	Odamaki *et al.*, 2005 [114]	Cross-sectional	141 HD pts	Aortic calcification area was significantly higher in HD pts with low fetuin-A levels.
7	Ziolkowska *et al.*, 2006 [230]	Cross-sectional	53 Children (18 pts with NS, 35 CKD) 22 healthy control	Fetuin-A concentration was lower in children with nephritic syndrome.
8	Honda *et al.*, 2006 [231]	Cross-sectional; Prospective (66 months)	176 CKD stage 5 pts	The prognostic power of fetuin-A for death did not differ significantly from serum IL-6, CRP, and S-albumin.
9	Cozzolino *et al.*, 2006 [232]	Cross-sectional	115 HD pts	Low serum fetuin-A was associated with high CRP, fibrinogen, and CV calcification score.
10	Coen *et al.*, 2006 [233]	Cross-sectional	38 HD pts	Fetuin-A and MGP levels correlated with bone formation parameters.
11	Coen *et al.*, 2006 [234]	Cross-sectional	132 HD pts	Low serum fetuin-A was associated with high CAC score.
12	Jung *et al.*, 2006 [235]	Prospective (2 years)	40 HD pts	Chronic inflammation and altered mineral metabolism, but not serum fetuin-A levels, contribute to progression of CAC.
13	Hermans *et al.*, 2006 [111]	Cross-sectional	131 Dialysis pts (98 HD, 33 PD)	Fetuin-A levels in dialysis pts did not differ from controls. Fetuin-A was not, in adjusted analysis, an independent predictor of aortic stiffness in dialysis pts with a low inflammatory activity.
14	Hermans *et al.*, 2007 [92]	Cross-sectional; Prospective (2.8 years)	987 Dialysis pts (664 HD and 323 PD)	High fetuin-A was associated with low CRP and high Ca levels. Fetuin-A was low in males and HD compared to females and PD pts. An increase in serum fetuin-A of 0.1 g/liter was associated with a 13% reduction in all-cause mortality and a 17% reduction in non-CV mortality.

(*continues*)

TABLE 1 (*Continued*)

	Study	Study type	Number and category	Conclusions
15	Russo *et al.*, 2007 [236]	Cross-sectional; Prospective (2 years)	53 CKD (stage 3–5)	Baseline fetuin-A did not differ significantly between pts with and without CAC, and fetuin-A did not predict CAC progression.
16	Caglar *et al.*, 2007 [237]	Cross-sectional	42 Tx pts	Low serum fetuin-A was associated with endothelial dysfunction.
17	Hermans *et al.*, 2007 [238]	Cross-sectional	134 Dialysis pts (103 HD, 31 PD)	Low serum fetuin-A correlated with intima-media thickness.
18	Cozzolino *et al.*, 2007 [239]	Cross-sectional	20 HD pts	A single HD session decreases serum fetuin-A, but not serum CRP, fibrinogen, or S-albumin levels.

HD=Hemodialysis; pts=patients; CKD=chronic kidney disease; CV=cardiovascular; PD=peritoneal dialysis; DM=diabetes mellitus; DN=diabetic nephropathy; OPG=osteoprotegerin; CAC=coronary artery calcification; Tx=kidney transplant; CRP=C-reactive protein.

high concentrations of fetuin-A were associated with the metabolic syndrome and atherogenic lipid profile [67]. Furthermore, recent reports from genetic studies suggest that single nucleotide polymorphisms in the *AHSG* gene are associated with adipocyte insulin action in humans [109] and with type-2 DM [110]. Recently, it has been demonstrated that a common SNP in the *AHSG* gene, associated with low fetuin-A, is more common among lean, rather than obese, men [107, 108]. Moreover, an SNP in the promoter region of *AHSG* gene was associated with insulin-mediated inhibition of lipolysis and the stimulation of lipogenesis in adipocytes [109]. Taken together, these findings suggest that fetuin-A is an important candidate among the factors that may induce insulin resistance and promote the metabolic syndrome.

6.7. Low Fetuin-A in Patients with CKD

The circulating fetuin-A level is low among patients with CKD in comparison with the general population. Consistent with results from Ketteler *et al.* [89], we have reported that the median concentration of fetuin-A was significantly lower in 258 CKD stage 5 patients starting dialysis treatment (0.225 g/liter; range 0.026–0.926 g/liter) compared to 70 healthy controls (0.549 g/liter; range 0.350–0.950 g/liter). This accords with the findings in most studies of CKD patients [90–92]. On the other hand, in one study of CKD stage 5 patients, Hermans *et al.* [111] reported that serum fetuin-A levels were not different from levels in healthy controls. However, in this CKD cohort [111], patients were characterized by a relatively low level of inflammatory activity. Also, Ix *et al.* [112, 113] have demonstrated in CAD patients and normal renal function that no relationship exists between fetuin-A concentration and degree of renal function within the normal interval. In another study of dialysis patients, Hermans *et al.* [92] found significantly higher fetuin-A concentrations in PD patients compared with HD patients. In contrast, we [90] did not find any difference in fetuin-A levels following 12 months of treatment with HD and PD, respectively.

6.8. Relations Between Fetuin-A, Inflammation, and Vascular Calcification

Inflammation and protein energy wasting (PEW) may be important causes of a decrease in serum fetuin-A levels in patients with CKD. Indeed, we found a significantly lower fetuin-A level in CKD stage 5 patients with evidence of inflammation and/or PEW, and fetuin-A [90]. Moreover, fetuin-A was inversely associated with both IL-6 and CRP and positively with S-albumin. The association between fetuin-A deficiency with low

S-albumin [91, 114] and other inflammation markers [89, 91] has been reported repeatedly in CKD patients.

Fetuin-A, like S-albumin, is predominantly a liver-derived and a negative acute-phase reactant. Hypoalbuminemia is strongly correlated to fetuin-A deficiency [91, 114] suggesting also an association with PEW and inflammation in CKD patients [90]. Although it has repeatedly been shown that low S-albumin levels are associated with both mortality [115] and cardiac disease [116, 117] in CKD patients, the reasons for this association has not been established. As we have found that the significant association between S-albumin and mortality was lost when fetuin-A was introduced into the Cox model [90], it could be speculated that the well-documented association between S-albumin and vascular disease may be explained, at least in part, by fetuin-A deficiency promoting vascular calcification.

We have also found a lower serum fetuin-A level in CKD stage 5 patients with PEW [90], a finding not surprising in view of the strong association between inflammation and PEW in CKD patients [118]. Similarly, Wang *et al.* [91] reported that low fetuin-A levels were associated with PEW and inflammation as well as with mortality and cardiovascular events in PD patients. Whether poor nutritional intake *per se* affects fetuin-A production in the liver remains to be determined. However, as we found no significant difference in fetuin-A levels comparing CKD patients with inflammation, defined as CRP $\geq$ 10 mg/liter, with and without signs of PEW, respectively [90], it is plausible that a persistent inflammation is the major cause of low fetuin-A levels also in wasted patients.

Several lines of evidence support the hypothesis that chronic inflammation may promote vascular calcification. First, as discussed above, fetuin-A is downregulated during inflammation [81, 119] and Wang *et al.* [91] have shown that fetuin-A was associated with cardiac valvular calcification and inflammation in PD patients, a finding that indirectly may link inflammation and the calcification process. Second, vascular calcification involves infiltration of monocytes and accumulation of macrophages. Tintut *et al.* [120] demonstrated that activation of monocytes and macrophages enhance *in vitro* vascular calcification via two independent mechanisms: cell to cell interaction and production of soluble factors, such as TNFα. In fact, TNFα may be an important promoter of vascular calcification by promoting osteoblastic differentiation of vascular cells through the cAMP pathway [98]. Third, the *ob* gene product leptin, which may be related to the presence of inflammation in CKD [121], has been shown to regulate osteoblastic differentiation and enhance the calcification of vascular cells [96]. Finally, Moe *et al.* [16] have demonstrated that OPN, which is secreted by macrophages and considered as an important regulator of inflammation, was strongly correlated with medial calcification and a history of CAD in patients

undergoing renal transplantation. Taken together, these findings suggest that vascular calcification is, indeed, a part of an active cell-mediated inflammatory process.

6.9. Low Fetuin-A Levels Are Associated with Poor Outcome in CKD

To this date, four studies have evaluated circulating serum fetuin-A levels in relation to all-cause and cardiovascular mortality in CKD patients [89–92]. Ketteler *et al.* [89] were the first group who reported low level of serum fetuin-A, which was associated with high CRP levels, in prevalent HD patients. In their cohort, patients within the lowest tertile of serum fetuin-A levels had a significantly higher all-cause and cardiovascular mortality compared to other two tertiles. Moreover, they showed in an *ex vivo* assay that the sera from patients with low fetuin-A concentration had a significantly impaired ability to inhibit $Ca\text{-}PO_4$ precipitation compared to sera with normal fetuin-A concentrations [89]. Subsequently, Stenvinkel *et al.* [90] confirmed (258 CKD stage 5 patients close to dialysis treatment) that low fetuin-A level was associated with all-cause and cardiovascular mortality independently of age, smoking, DM, S-albumin, CVD, and inflammation. In this study, low fetuin-A level was also associated with carotid plaque formation [90]. Recently, in the largest dialysis cohort studied so far, Hermans *et al.* [92] showed that serum fetuin-A deficiency is a predictor of both cardiovascular and noncardiovascular mortality. Although these studies [89, 90, 92] did not directly address the link between fetuin-A levels and the magnitude of vascular calcification in CKD patients, they consistently show the associations between low fetuin-A levels and inflammation, accelerated atherosclerosis and CVD mortality in CKD patients. Adding to these results, Wang *et al.* [91] showed that low fetuin-A is associated with valvular calcification, atherosclerosis, and predicted mortality and cardiovascular events in 238 PD patients. In a small cohort of CKD patients, Moe et al. [122] showed a significant association between coronary calcification and fetuin-A deficiency. Moreover, Odamaki *et al.* [114] found in 141 Japanese HD patients that the aortic calcification area was significantly higher in patients with low fetuin-A than in those with high fetuin-A. The latter showed a significantly negative correlation with carotid plaques. These findings in CKD patients accord with a recent report in non-renal patients. Ix *et al.* [112] demonstrated associations between calcifications of mitral and aortic valves with low serum fetuin-A levels in 970 patients with CAD (Table 2).

However, not all studies have been able to demonstrate a link between serum fetuin-A and markers of vascular calcification. As an example, Hermans *et al.* [111] reported that in CKD patients with a relatively low level of inflammatory activity, serum fetuin-A could *not* be identified as an

TABLE 2
MAJOR FINDINGS IN THE CLINICAL STUDIES ON FETUIN-A IN NON-RENAL POPULATIONS

	Study	Study type	Number and category	Conclusions
1	Kalaby *et al.*, 1990 [240]	Cross-sectional	63 pts with SLE	Serum fetuin-A concentration was lower in SLE pts.
2	Kalaby *et al.*, 1992 [87]	Cross-sectional	753 pts with various diseases	Whereas the concentrations of serum fetuin-A were low in variety of diseased, it was elevated in pts with B and C hepatitis.
3	Kalaby *et al.*, 2002 [88]	Cross-sectional; Prospective (1 month)	191 pts with liver disease and liver cancer	Patients with liver cirrhosis and hepatocellular cancer, but not those with acute viral hepatitis, had low serum fetuin-A concentrations. Low fetuin-A levels were associated with increased mortality.
4	Mathews *et al.*, 2000 [102]	Prospective (7 days)	20 pts with AMI	Serum fetuin-A levels were low in AMI pts. Fetuin-A concentrations decreased within a few hours after the onset of AMI.
5	Ix *et al.*, 2006 [113]	Cross-sectional	970 outpatients with CAD (CKD stage 1–3)	No evidence that mild-to-moderate CKD is associated with lower concentrations of serum fetuin-A among individuals with CAD. Independent of kidney function, serum fetuin-A was associated with serum Ca and PO_4 concentrations.
6	Ix *et al.*, 2006 [67]	Cross-sectional	711 Nondiabetic outpatients with CAD	High fetuin-A concentration was independently associated with metabolic syndrome and an atherogenic lipid profile.
7	Mori *et al.*, 2006 [241]	Cross-sectional	321 (161 type-2 DM) subjects	No differences in fetuin-A concentrations between nondiabetic and type-2 diabetic individuals. High serum fetuin-A concentration was independently associated with insulin resistance (HOMA) in nondiabetic subjects. However, no significant relationship was observed between fetuin-A levels and insulin resistance in diabetic subjects.

8	Stefan *et al.*, 2006 [106]	Cross-sectional	106 Healthy subjects	A high serum fetuin-A level was independently associated with insulin resistance (euglycemic-hyperinsulinemic clamp) and with fat accumulation in the liver.
9	Hendig *et al.*, 2006 [242]	Cross-sectional	110 PXE pts	Serum fetuin-A concentrations in PXE pts were lower than in unaffected first-degree relatives and healthy controls. Fetuin-A polymorphism frequencies did not differ among PXE pts, family members, and healthy controls.
10	Fiore *et al.*, 2007 [124]	Cross-sectional	90 pts with atherosclerotic plaques	High serum fetuin-A levels were associated with intima-media thickness. Fetuin-A levels were significantly higher in pts compared to subjects with no plaques.
11	Ix *et al.*, 2007 [112]	Cross-sectional	970 Outpatients with CAD (26% diabetic)	High serum fetuin-A concentration was associated with less mitral annular calcification (OR 0.47). This association was similar regardless of diabetes status. Similar association was also observed between high fetuin-A and presence of aortic stenosis in nondiabetic individuals (OR 0.37); whereas among diabetics no association was observed between fetuin-A and aortic stenosis.
12	Mori *et al.*, 2007 [243]	Cross-sectional	141 Healthy subjects	High serum fetuin-A level was independently associated with carotid arterial stiffness.
13	Kaden *et al.*, 2007 [244]	Cross-sectional	59 Subjects with (n=31) or without (n=28) calcific aortic stenosis	Serum fetuin-A levels were lower in pts compared to healthy controls. Immunohistochemistry staining of fetuin-A was found in stenotic valves but not in healthy control valves.
14	Dahlman *et al.*, 2004 [109]	Cross-sectional	364 Healthy women (188 lean and 176 obese)	The *–469T>G* SNP of *AHSG* gene was associated with insulin-mediated inhibition of lipolysis and stimulation of lipogenesis. Three *AHSG* SNPs were associated with cholesterol. None of six *AHSG* SNPs were associated with BMI, body fat mass, waist circumference, glucose, or insulin resistance.
15	Osawa *et al.*, 2005 [133]	Cross-sectional	176 Healthy subjects	The *AHSG2* allele is associated with lower levels of serum fetuin-A compared to *AHSG1* allele.

(*continues*)

TABLE 2 (*Continued*)

	Study	Study type	Number and category	Conclusions
16	Lavebratt *et al.*, 2005 [107]	Cross-sectional	93 Healthy men	A common variation (Thr230Met) in the *AHSG* gene is markedly associated with increase in β2-adrenoceptor sensitivity in subcutaneous adipose tissue.
17	Lavebratt *et al.*, 2005 [108]	Cross-sectional	504 Healthy subjects (356 overweight or obese and 148 lean	A common variant, *rs2593813*:G–230:Met–238:Ser, of the insulin receptor inhibitor gene *AHSG* is more common among lean than among obese and overweight Swedish men suggesting that a low level of fetuin-A is protective against obesity.
18	Lehtinen *et al.*, 2007 [132]	Cross-sectional	829 Type-2 DM pts	Four SNPs of *AHSG* gene were independently associated with CAC.

SLE=Systemic lupus erythematosus; AMI=acute myocardial infarction; CAD=coronary artery disease; PXE=pseudoxanthoma elasticum disorder; HOMA=Homeostatis model assessment; BMI=Body mass index. Other abbreviations as in Table 1.

independent predictor of aortic stiffness as measured with pulse-wave velocity. Furthermore, in diabetic CKD stage 1–4 patients, with or without diabetic nephropathy, high, rather than low, fetuin-A levels were positively related to the magnitude of coronary artery calcification (CAC) [123]. Also in another study performed in non-renal population with evidence of atherosclerotic plaques, fetuin-A concentration was higher in patients compared to age- and gender-matched healthy subjects and positively correlated with the severity of peripheral vascular calcification [124]. It is notable that also in this study patients with atherosclerosis were characterized by a low level of inflammatory activity. The authors speculated that low grade of inflammation in their patients leads to a negative bone remodeling balance due to reduced bone formation and consequently prevent incorporation of fetuin-A into bone [124]. Instead fetuin-A is shifted to serum where it is incorporated in serum mineral complex generated as a consequence of the inhibition of bone mineralization [74]. It is clear that the relationship of fetuin-A with extraosseous calcification, including vascular calcification, is divergent in CKD patients, which may reflect its diverse actions.

Apart from its function in bone, fetuin-A promotes insulin resistance [101, 125] and interferes with insulin receptor phosphorylation [102], as mentioned previously. In a recent study of 711 nondiabetic outpatients with CAD participating in the Heart and Soul Study, Ix *et al.* [67] found that a high circulating fetuin-A concentration was associated with the metabolic syndrome, even after adjustment for potential confounding variables. In the same study, higher fetuin-A was also associated with higher low-density lipoprotein (LDL)-cholesterol and triglyceride concentrations, and lower high-density lipoprotein (HDL)-cholesterol concentrations [67]. This dual role of fetuin-A may explain the discrepancy in the relationship between fetuin-A and aortic valve calcification among the diabetic and nondiabetic patients [112]. In line with this assumption, whereas prior epidemiological studies in populations primarily without DM have demonstrated an inverse correlation of fetuin-A with vascular calcification [82, 122], there is a direct correlation in DM population [123]. It is possible that factors inducing an insulin-resistant state may result in high serum fetuin-A levels, which may be protective in this setting by limiting the amount of dystrophic calcification conferred by an insulin-resistant state [126, 127]. The relation of fetuin-A to calcification seems to be different in diabetic patients with more or less impaired renal function (CKD stage 1–4) [123]. Thus, in patients with diabetic nephropathy a positive association between fetuin-A levels and coronary calcification scores was demonstrated [123]. Based on these findings it

has been hypothesized that fetuin-A upregulation is a systemic feedback defense mechanism protecting against vascular calcifications in the early stages of diabetic nephropathy [128].

Whether fetuin-A upregulation may serve as a systemic defense mechanism and a physiological attempt to counteract early vascular calcifications remains to be investigated. Indirect support for this speculation may be the recent immunohistochemical findings showing markedly increased fetuin-A depositions around areas of vascular calcification, while the protein is not expressed locally [122]. However, patients in late stages of kidney dysfunction could potentially develop progressive fetuin-A deficiency due to exposure to high levels of uremic toxins or to persisting low-grade inflammation. Moreover, during dialysis treatment more extreme calcification develops [123], which may exhaust the fetuin-A system and consume existing fetuin-A from the circulation, collectively causing depletion of fetuin-A in the serum. Indeed, in the prospective part of the study by Stenvinkel *et al.* [90] it was shown that one year of dialysis treatment was associated with a small, but significant, reduction in serum fetuin-A levels. This finding may indirectly support the suggestion that the process of vascular calcification is accelerated by dialysis treatment. Indeed, whereas Spiegel *et al.* [129] reported that coronary and aortic calcification was not a very common phenomenon in patients new to HD treatment, Ketteler *et al.* [89] reported higher coronary calcification scores in patients on long-term dialysis compared to short-term dialysis. Moreover, Moe *et al.* [130] demonstrated that duration of dialysis (vintage) and age were the only factors that correlated with coronary calcification. Thus, it is tempting to speculate that exhaustion of fetuin-A concentrations during dialysis treatment may be one reason for the accelerated vascular calcification process in this patient group. As neither mode of dialysis therapy nor changes in S-albumin or CRP levels were associated with the observed decline in fetuin-A levels [90], further studies are needed to investigate if other factors, such as changes in residual renal function, acid–base balance, or nutritional status, may cause a decline of fetuin-A levels during dialysis. We have to bear in mind that whereas calcification is a slowly progressive process over time with an unknown starting point, fetuin-A levels may fluctuate substantially, possibly dependent on repeated flares of inflammation. Clearly, further studies in larger cohorts with multiple longitudinal observations and serum measurements are probably to delineate these relationships. So far, current data suggest the concept that fetuin-A, as a calcification inhibitory factor, may progressively fail with the development of uremia due to yet unidentified mechanisms and fetuin-A could be considered as a local biomarker of the calcification burden as well as a systemic marker of active disease.

6.10. Fetuin-A and Genetic Polymorphisms

Evidence supporting the anticalcific role of fetuin-A first came from studies with deficient mice showing increased susceptibility to both blood vessels and soft tissue calcifications. Indeed, Schäfer *et al.* [20] have demonstrated that deletion of the fetuin-A gene (fetuin-$A^{-/-}$) in mice causes a phenotype of extraosseous calcification of various organs, including kidney, testis, skin, heart, and vasculature. This depends on the genetic background of mouse as DBA/2 mice calcify spontaneously whereas C57Bl/6 mice need additional stimuli like vitamin D treatment in order to show calcifications [20]. Several nonsynonymous polymorphisms exist in the *AHSG* gene [131]. It is plausible that genetic alterations may have an effect on the circulating amounts of this protein also in human. Stenvinkel *et al.* [90] were able to identify a specific fetuin-A gene polymorphism (*AHSG* 256Ser allele) which had lower serum fetuin-A levels and a significantly worse outcome than those patients carrying the alternative polymorphisms. In accordance, Lehtinen *et al.* [132] found that polymorphisms in four SNPs in AHSG were associated with coronary artery calcified plaques in 829 European American subjects with type-2 DM. Of note, the presence of inflammation had an inhibitory effect on fetuin-A levels in patients carrying the *AHSG* 256Thr allele, a finding suggesting a significant gene-environment interaction, where the involvement of inflammatory processes may further enhance the negative effects of low circulating fetuin-A levels [90]. Another report by Osawa *et al.* [133] demonstrated associations of an *AHSG* gene polymorphism (*AHSG2* allele) with lower serum fetuin-A levels and with serum PO_4 levels in the general population. The association of this gene polymorphism with PO_4 is interesting, although it is difficult to interpret since it was observed in healthy subjects in whom serum PO_4 is predominantly regulated by renal excretion and bone turnover, both controlled by PTH and vitamin D. It is yet unknown whether fetuin-A has any impact on the regulation of PTH and vitamin D, or has a role on renal, gastrointestinal, or osseous functions with regard to phosphate handling. Clearly, additional studies are needed to evaluate if AHSG 256Ser allele or *AHSG2* allele carriers are a group of CKD patients particularly prone to develop vascular calcification, and would benefit from early detection.

Furthermore, Lavebratt *et al.* [107, 108] demonstrated that a common SNP in the *AHSG* gene associated with lower circulating AHSG is more common among lean than obese and overweight men, suggesting a link between these gene mutations and a role for AHSG in the metabolic syndrome. Moreover, an SNP in the promoter region of AHSG was associated with insulin-mediated inhibition of lipolysis and the stimulation of lipogenesis in adipocytes [109]. Clearly, further studies should be performed to evaluate these and other pathways linking fetuin-A with adipose tissue biology in CKD.

7. Matrix-Gla Protein

MGP is the first recognized protein that acts as a calcification inhibitor *in vivo* [134]. It is a 10-kDa protein containing five γ-carboxyglutamic acid (GLA) residues expressed in several tissues, including bone, kidney, lung, heart, cartilage, and VSMCs of the blood vessel wall [134, 135]. MGP expression increases with increase in extracellular Ca concentrations and it is also increased in atherosclerotic plaques [21, 136, 137]. It inhibits both arterial and cartilage calcifications. However, it also inhibits mesenchymal cell differentiation to the osteogenic lineage by sequestering the potent osteogenic and chondrogenic differentiation factor, BMP-2. The effect of MGP and BMP-2 depends on the degree of MGP γ-carboxylation and the ratio of the levels of these two molecules, suggesting that lack of function of MGP, rather than its amount, may be the factor that increases the risk of calcification [21, 138, 139]. The local increase of MGP expression during vascular calcification may limit the extent of calcification because MGP can bind to BMP-2 [140, 141]. MGP is by virtue of its GLA residues a Ca-binding protein. Thus, a potential mechanism of MGP action in inhibiting Ca-PO_4 deposition is Ca chelation [142]. Alternatively, circulating complexes of MGP and Ca-PO_4 have been identified, suggesting that MGP is involved in Ca-PO_4 clearance [74] and it also binds to elastin [97]. In contrast to its mineral-binding properties, MGP was also found to inhibit BMP-2 activity via matrix association and may thus inhibit osteogenic differentiation [138]. Although the role of MGP in calcification is complex, it is evident that MGP is a major inhibitor of both arterial and cartilage calcification and regulates bone and vascular homeostasis.

Mounting evidence from animal studies underline the role of MGP in normal vascular function. Knockout mice experiments demonstrate that MGP acts *in vivo* as an inhibitor of mineralization in arteries and cartilage and MGP-deficient mice are subjected to dramatic ectopic calcifications [143]. In these mice, abnormal cartilage calcification and extensive calcification of elastic arteries developed within a few weeks. The calcification caused premature death due to rupture of the aorta [143]. Moreover, a rare autosomal recessive condition (Keutel syndrome) due to mutations in the gene encoding MGP [144] is associated with calcification of cartilage, but not arteries. However, unlike the mouse model, Keutel syndrome patients survive into adulthood, which implies that also other factors may function to inhibit vascular calcification in humans. In MGP-deficient mice, the vascular calcification starts in the media and, once initiated, spreads rapidly along elastin fibers. However, there is no evidence of atherosclerotic lesions or ectopic bone formation in these mice [143].

As MGP is a member of the vitamin K-dependent protein family this protein needs vitamin K-dependent γ-carboxylation enzyme for its biological activation. Thus, in the case of deficiency of functional vitamin K, calcification may be enhanced owing to decreased availability of functional of MGP [21]. An association between vascular calcification and lower vitamin K intake was found in postmenopausal women [145]. In accordance, the large population-based Rotterdam study showed a reduced CAD risk with increasing amounts of dietary vitamin K intake [146]. Although poor nutrition is a common finding in CKD patients, the effect of vitamin K supplementation on vascular health has, to the best of our knowledge, not been studied. Also, warfarin interferes with the availability of bioactive vitamin K and may interfere with MGP function. As inhibition of the γ-carboxylation of Gla residues with warfarin in both cell culture experiments and *in vivo* studies resulted in increased matrix mineralization, this suggests that the mineral-binding Gla residues of proteins, such as MGP, are crucial for the regulation of matrix mineralization [147, 148]. In accordance, treatment with warfarin at doses that inhibit the vitamin K-dependent γ-carboxylation of MGP induces progressive calcification of arterial media and increases gene expression of MGP in the calcifying artery in the rat [147, 148]. The potential clinical importance of vitamin K-dependent γ-carboxylation is underlined by recent studies showing that patients on oral anticoagulant therapy had increased coronary and valvular calcifications compared to patients without anticoagulation treatment, presumably due to less active MGP [149, 150]. This issue may have important clinical implications for the use of vitamin K antagonist-based anticoagulation substances like warfarin in both non-renal and renal patient populations. However, although high doses of warfarin can cause vascular calcification in rats, such effects on calcification in man have not been described although one study showed that warfarin was a risk factor for the development of the life-threatening condition calciphylaxis in CKD patients [151].

A relation between serum MGP concentrations and artery calcification has been reported in the rat with threefold increase in MGP levels in animals with the greatest artery calcification [147]. This is presumably secondary to increased local synthesis of MGP for the purpose of slowing the progression of artery calcification. However, increased serum levels of MGP, without a concomitant increase in MGP expression in the arterial walls, does not inhibit the ectopic mineralization observed in mice lacking MGP [152]. The association between serum MGP and vascular calcification seems controversial in the light of the findings in the general population. For example, it has been reported that serum MGP levels were inversely associated with severity of CAC detected by electron beam computer tomography (EBCT) in 115 patients with CAD [153]. Whereas, another study showed that serum MGP

concentration was significantly high in patients with severe atherosclerosis and the levels were normal in individuals with low bone mass and osteoporosis [154]. Also, a recent study by O'Donnell *et al.* [155] reported that MGP level is associated with atherosclerosis risk factors but not with CAC.

The relationships between MGP and established atherosclerosis risk factors are unclear, and it is uncertain whether risk factors individually or together may confound the relationship between MGP and CAC. It is also possible that other confounding factors may underlie these differing findings. Nonetheless, the fact that MGP knockout mice (MGP $^{-/-}$) develop extensive vascular calcification and die prematurely of a ruptured aorta [143] supports the concept that MGP has significant inhibitory role in calcification. Of note, in a clinical study performed in a low-risk male population, MGP-7 AA homozygotes have been shown to have an almost four times higher risk of myocardial infarction [156]. Moreover, AA homozygotes exhibited femoral artery calcification in the presence of femoral atherosclerotic plaque more frequently than subjects with other genotypes [156]. Also, MGP-7 AA homozygosity, compared to other genotypes, was found to be associated with a higher level of left ventricular hypertrophy and accelerated progress of atherosclerosis in one year, based on carotid artery ultrasound assessments, [157]. In accordance, Brancaccio *et al.* [158] showed that nondialyzed CKD patients and HD patients who are MGP–138TT homozygotes and MGP–7AA homozygotes have a significantly worse prognosis and cardiovascular events.

8. Osteoprotegerin

OPG is a circulating glycoprotein that serves as a decoy receptor for the TNF ligand superfamily member RANKL [159] and TNF-related apoptosis-inducing ligand [160, 161]. It is a secreted TNF-receptor-like molecule and acts as an inhibitor of terminal differentiation and activation of bone-resorbing osteoclasts and, thus, serves as a potent inhibitor of bone resorption *in vivo*. Unlike most members of the TNF receptor superfamily (which are transmembrane proteins), OPG is a secreted protein [160]. It binds to osteoprotegerin ligand (OPGL) and thereby inhibits the interaction between receptor activator of nuclear factor (NF)-κB (RANK) and OPGL on osteoclasts differentiation [162]. OPG-deficient mice exhibit a decrease in total bone density, marked thinning of the parietal bones of the skull, a high incidence of fractures, and also medial calcification of the aorta and renal arteries [163]. Thus, OPG seems to be of importance both in osteoclastogenesis and vascular calcification. Transgenic overexpression of OPG in mice produces an osteopetrotic phenotype due to the inhibition of growth-related

bone resorption [122]. Disruption of the *OPG* gene produces osteoporosis marked by excessive bone resorption indicating the importance of this molecule in normal bone physiology. OPG, a RANK homolog, works by binding to the RANK-ligand on osteoblast/stromal cells, thus blocking the RANK–RANK ligand interaction between osteoblast/stromal cells and osteoclast precursors. OPG is found in a number of tissues including the major arteries [160]. It is also highly expressed in VSMCs, where it is upregulated by platelet-derived growth factor, fibroblast growth factor, angiotensin II, TNFα, and IL-1β [164]. Emerging evidence indicates that OPG is not only a bone protector, but also acts as a protective factor for the vascular system [165]. A recent study demonstrated OPG and RANKL immunoreactivity in early atherosclerotic lesions in human tissues, suggesting a regulatory role of these proteins not only in osteoclastogenesis but also in atherosclerotic calcification [166]. In a rat model, warfarin-induced vascular calcification was prevented with OPG treatment [162]. However, recombinant OPG, which known to protect against osteoporosis, did not reverse vascular calcification in OPG-deficient mice [167]. In contrast, the same study showed that OPG transgenic mice delivered from midgestation to adulthood did prevent arterial calcification [167], suggesting that although OPG can help prevent arterial calcification, it cannot reverse it. Similarly, MGP null mice develop severe medial vascular calcification and die at a one month age from CAD and vascular aneurysms [143].

Although the mechanism(s) whereby OPG prevent arterial calcification is still unclear, human studies have shown a paradoxical positive association between serum OPG levels and CAC [122]. OPG levels were 30% higher in women with DM and increased OPG levels were associated with all-cause and cardiovascular mortality [168]. Also, in patients who underwent coronary angiography, OPG level was greater in patients with significant coronary stenosis compared with those without stenosis and the increase in OPG level was associated with the severity of disease [169]. These findings were later confirmed by another study in 522 men undergoing coronary angiography [170]. Moreover, an association between OPG concentration and progressive atherosclerosis has been reported [171].

As increased levels of OPG in CKD patients [172, 173] are associated with serum creatinine levels and 24-hour creatinine clearance [173], the kidney seems to be a major site for clearance of OPG. In a prospective study, Nitta *et al.* [174] showed that the rapid progression of vascular calcification was associated with serum OPG concentration in a group of HD patients. Moreover, the same investigators reported that the aortic calcification index in HD patients was associated with OPG levels [100]. Thus, increased levels of OPG predict all-cause and cardiovascular mortality in both dialysis [175] and renal transplant [176] patients. Recently, Morena *et al.* [175] showed that high

OPG levels were associated with all-cause and cardiovascular mortality and that these associations were stronger in inflamed HD patients. As increased levels of serum OPG are associated with increased cardiovascular risk, these findings seem to contradict the calcification inhibitory properties of this protein. Based on these paradoxical findings it could be speculated that increased OPG may result from enhanced synthesis in vessel walls by activated osteoblast-like cells, committed to vascular calcification.

As the process of calcification includes monocyte and macrophage infiltration, the release of pro-inflammatory cytokines, such as TNFα, may also affect vascular calcification by promoting osteoblastic differentiation through the cAMP pathway [98]. Results in non-renal patients suggest that polymorphisms within the TNFα gene may have an impact on the calcification phenotype [177]. Although OPG knockout animals develop both osteoporosis and vascular calcifications [163], serum OPG levels were significantly elevated in HD patients with a higher aortic calcification index [100]. Therefore, it can be speculated that increased serum levels of OPG may be a compensatory protective response to the progression of vascular calcification. Moe *et al.* [16] demonstrated that vascular calcification of the medial layer of epigastric arteries is associated with deposition of OPG in CKD patients undergoing renal transplantation. These data are corroborated by the recent findings of Strom *et al.* [178] in triple knockout mice (ApoE/LDL-receptor/OPG) showing that OPG deficiency reduces atherogenesis. Several functional *OPG* gene polymorphisms exist, and in Caucasian men, the genotype combinations 950 TC/1181 GC and 950 CC/1181 CC may confer an increased risk of CAD [179]. Although *OPG* gene polymorphisms have been associated with osteoporosis and CVD in non-renal patients [179], its significance in the extraosseous calcification process in CKD patients remains largely unexplored.

9. Osteopontin

OPN is a single-chain polypeptide glycoprotein with a molecular weight of approximately 32,600 kDa first identified in osteoblasts in 1986 [180]. OPN is an extracellular structural protein and an organic component of bone, which functions as an important calcification inhibitor. Synthesis of OPN is stimulated by calcitriol in a variety of tissues and in a variety of cancers. OPN is an acidic phosphoprotein normally present in skeleton, where it facilitates the attachment of osteoclasts to the bone matrix via an interaction with cell surface $\alpha v\beta 3$ integrin and CD44, the hyaluronic acid receptor [181]. OPN is a potent inhibitor of vascular calcification via direct inhibition of apatite

growth by binding to crystal surfaces and induction osteoclast function through $\alpha v\beta 3$ integrin (26). It also promotes the expression of carbonic anhydrase II in monocytes and increases the acidification of the extracellular milieu [182]. It is known that carbonic anhydrase deficiency leads to soft tissue calcifications and osteopetrosis [183]. Therefore, OPN inhibits vascular and soft tissue calcifications through direct and indirect pathways. OPN directly inhibits calcification of cultured bovine aortic smooth muscle cells and inhibits aortic valve calcification *in vivo* [21, 182, 184]. In CKD patients, hyperphosphataemia is associated with the expression of OPN in VSMCs [185]. Mice with a genetic deficiency of MGP and OPN have accelerated aortic calcification compared with mice deficient in MGP alone; consistent with the concept that OPN inhibits mineralization [21, 186]. OPN produced locally by VSMCs may serve as an important inducible inhibitor of vascular calcification [187]. It has been reported that OPN is abundant at sites of calcification in human atherosclerotic plaques and in calcified aortic valves but is not in normal arteries [188–194]. In HD patients, OPN levels were significantly higher compared to healthy subjects and correlated with aortic calcification index [195]. In the same study, Nitta *et al.* [195] reported that OPN is abundantly expressed in the atherosclerotic lesions of aortic tissues and that this expression was related to the degree of atheromatous plaques in HD patients. Similar to OPG, this suggests that elevated OPN levels are a counterregulatory response to the increased calcification burden in CKD and limit the extent of vascular calcification.

OPN acts as a cytokine and is secreted by activated macrophages, leukocytes, and activated T lymphocytes at sites of inflammation [196, 197]. This cytokine mediates important cell–matrix and cell–cell interactions. In the immune system, OPN plays a role in chemotaxis, leading to the migration of macrophages and dendritic cells to sites of inflammation. Activation of T lymphocytes results in an increase in OPN transcription. It has been demonstrated that OPN is a T-lymphocyte suppressor factor and enhances B-lymphocyte immunoglobulin production and proliferation [198]. In addition, OPN is an important cytokine mediating Th1 immunity [199]. Thus, OPN is a multifunctional protein expressed by many cell types, with roles in atherosclerosis, cell-mediated immunity, and macrophage recruitment and activation [200]. In early stages of atherosclerosis, OPN attracts inflammatory cells, promotes the release of proteolytic enzymes, and stimulates VSMCs proliferation [201, 202]. Plasma OPN is increased in severe CAD [203], and there are high concentrations of OPN found in the intima from carotid endarterectomy specimens [204]. In later stages of atherosclerosis, macrophages synthesize OPN at high levels, which may limit further calcification [205].

10. Bone Morphogenetic Protein-7

BMPs are secreted signaling molecules that comprise a subfamily of the TGFβ superfamily of cytokines. Originally identified as protein regulators of cartilage and bone organs, it has been shown that BMPs are also involved in embryogenesis and morphogenesis of various tissues and organs. They play an essential role in pattern formation during embryogenesis, control osteoblast differentiation, and promote osteogenesis [206]. There are at least 20 structurally and functionally related BMPs, most of which play roles in embryogenesis and morphogenesis of various tissues and organs.

The effects of local BMP are mediated by intracellular regulators that include stimulatory and inhibitory Smad proteins, of which Smad6 is an inhibitor of BMP signaling [207]. The basic signal mechanisms consist of two receptor serine/threonine protein kinases (receptor types 1 and 2) and a family of receptor substrates (the Smad proteins) that move into the nucleus [208]. Targeted mutation of *Madh6* (the gene that encodes Smad6) in mice showed an important role of Smad6 in the development of the cardiovascular system [209]. Smad6-deficient mice had cardiac valve and outflow tract defects and suffered from considerable perinatal mortality [209]. Moreover, their heart valves were hyperplastic, the septum appeared misplaced, with aorta showing cartilaginous metaplasia and ossification of the medial layer. This may lead to impairment of vascular relaxation and increase of the mean arterial blood pressure [209], it could be suggested that Smad6 limits the osteogenic responsiveness of the cardiovascular system to TGF/BMP signals [209].

BMP-7 is also essential for the development of kidneys, eyes, and bones [210]. In the adult, BMP-7 maintains a role in osteoblast function, suggesting a hormonal role in bone metabolism. BMP-7 expression decreases early in the course of renal failure [211] and recent findings suggests that BMP-7 is involved in the process of vascular calcification of CKD and in the pathogenesis of renal osteodystrophy [99, 212, 213]. Thus, BMP-7 deficiency may have important roles in the pathogenesis of CKD [214] and could also influence the pathogenesis and treatment of vascular calcifications. It has been shown that BMP-7 maintains VSMCs differentiation and prevents their transformation into an osteoblastic phenotype [212, 215]. Transdifferentiation of VSMCs in osteoblastic phenotype could be the critical first step in the cause of vascular calcification and it is clear that BMP-7 has a positive influence in maintaining VSMC differentiation. Because vascular calcification in CKD appears to be regulated in a process similar to bone formation, one considers a role of BMPs in the pathogenesis of vascular calcification. However, although the roles of the BMPs in osteogenesis are well documented, their roles in vascular calcification are more complex and

less well defined. Whereas BMP-7 appears to inhibit vascular calcification, BMP-2 seems to be a strong basic causative factor in vascular calcification [216]. Thus, further studies defining the precise role of BMPs in vascular calcification are needed, particularly with regards to how factors with similar signaling cascades can have such divergent effects. Davies *et al.* [99] showed that BMP-7 is an effective treatment of vascular calcification in the context of a murine model of atherosclerosis and CKD, a finding that may have important implications for the development in human beings of future therapies for this condition, which currently is without treatment and with strong negative influences on cardiovascular morbidity and mortality.

11. Inorganic Pyrophosphate

PPi is emerging as one of the potent physiological inhibitors of hydroxyapatite formation and considered as a potent inhibitor of vascular calcification and increasingly recognized as a vascular paracrine factor that mediates this process [217]. Pyrophosphate is generated as a by-product of various metabolic reactions, and the levels are maintained by the activity of three compounds: nucleotide pyrophosphatase phosphodiesterase-1 (NPP-1), multiple-pass transmembrane protein encoded by the *progressive ankylosis* locus (ANK), and tissue nonspecific alkaline phosphatases (TNAP). Intracellular synthesis of PPi is regulated by the rate-limiting enzyme ENPP-1 and the appropriate transport of PPi into the extracellular environment is controlled by ANK where the excess of extracellular PPi is degraded by TNAP to phosphate. Accordingly, activity of NPP-1 and ANK prevents ectopic calcification, while high TNAP activity favors calcification by increasing PO_4 concentrations. Thus, the mechanism by which pyrophosphate modulates vascular calcification is through direct physiochemical inhibition of hydroxyapatite formation and preventing propagation of tissue Ca deposition [218]. Nonetheless, deficiencies in pyrophosphate lead to deficiency in OPN expression, another calcification inhibitor, in mouse primary osteoblasts [219], which may suggest another mechanism in this process. Deficiency in NPP1 is associated with vascular calcification whereas decreased ANK promotes endochondral vascular calcification [220–222]. Creating ANK deficiency in transgenic mice leads to the particular phenotype of periarticular hydroxyapatite deposition and progressive inflammatory arthritis [223]. Moreover, PPi is a direct and potent inhibitor of hydroxyapatite formation *in vitro*, and even small concentrations in plasma are sufficient to completely prevent crystallization from saturated solutions of Ca and PO_4 [224–226]. In idiopathic infantile arterial calcification, a human genetic disease, deficiency of PPi, due to deficient NPP-1 activity, is associated with widespread

calcification of the blood vessels and articular cartilage and usually results in mortality by 6 months of age [220]. Moreover, low levels of PPi as a result of the absence of NPP-1 enzyme lead to severe, fatal arterial calcification that can be prevented by therapy with bisphosphonates [227, 228]. So far, *in vivo* and *in vitro* findings suggest that vascular calcification cannot occur in the presence of normal concentrations of PPi and that the vascular calcification in CKD may be associated with altered PPi metabolism. A recent study reported low plasma PPi levels in HD patients that are exacerbated as a result of dialysis clearance [229]. Thus, abnormalities in pyrophosphate metabolism in CKD patients might also help explain the predominance of vascular calcification in such patients. Whether low levels of PPi are associated with vascular calcification in CKD remains to be determined.

12. Conclusion

The annual mortality rate in CKD patients is extremely high primarily due to cardiovascular events. Apart from traditional Framingham risk factors, a growing body of evidence points to nontraditional risk factors, including disturbances in mineral metabolism. Disturbances in mineral metabolism, especially Ca and PO_4, have been linked to vascular calcification. Vascular calcifications play a vital role in the development of cardiovascular events and subsequent increased mortality. With the relatively recent recognition of vascular calcification as an actively regulated process and the consequences of the active and dynamic balance of procalcifying and anticalcifying influences, growing numbers of calcification inhibitors (fetuin-A, MGP, OPG, OPN, BMP-7, and PPi) have been discovered. Recent evidence suggests that alterations of these inhibitors may contribute to the high prevalence of vascular complications in CKD patients. Although our understanding of the physiological and pathophysiological roles of calcification inhibitors is currently still in the early stages, it is likely that fetuin-A is one of the most relevant factors among an increasing number of other calcification inhibitors. Both chronic inflammation and the uremic milieu *per se* may contribute to an exhaustion of fetuin-A in CKD. Specific mutations of the *AHSG* gene may be additional determinants of fetuin-A deficiency. The emerging role of fetuin-A deficiency as a risk factor is documented in CKD demonstrating a significant correlation with vascular calcification, all-cause and cardiovascular mortality. Thus, based on the growing research in the field of vascular calcification, it can be expected that novel diagnostic and therapeutic approaches considering deficiencies of calcification inhibitors may develop in the near future and help to abate and potentially reverse the deleterious of vascular calcification and death.

Acknowledgments

This work was supported by Swedish Heart and Lung foundation (P.S.), Swedish Medical Research Council (P.S.), and Martin Rinds Foundation (P.S.).

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MECHANISMS OF ARTERIAL CALCIFICATION: SPOTLIGHT ON THE INHIBITORS

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0065-2423/08 $35.00
DOI: 10.1016/S0065-2423(08)00407-1

1. Abstract

Similarities in the mechanisms of vascular calcification and the processes of bone and cartilage mineralization have come to light in recent years. Although formerly thought to be an inactive process of hydroxyapatite crystal precipitation, presently, vascular calcification is considered a regulated type of tissue mineralization. Moreover, different pathways of tissue mineralization are discussed. Pathological types of calcification are correlated with aging, metabolic disorders, chronic low-grade inflammation, and with genetic and acquired dysregulation of inorganic pyrophosphate (PP_i) metabolism. This chapter focuses on recent developments in understanding the mechanisms of vascular calcification with special emphasis on the particular calcification pathway and the impact of deficient inhibition of calcification.

2. Introduction

Vascular calcification occurs with atherosclerosis, valvular disease [1, 2], and varicosis [3]. Advanced age and metabolic disorders, including end-stage renal disease (ESRD) and diabetes mellitus, are known contributing factors. Genetic disorders associated with vascular calcification include connective tissue diseases and abnormalities of phosphate metabolism. Arterial calcification contributes to hypertension and an increased risk of cardiovascular events [4] and correlates with an increased frequency of ischemic episodes in peripheral vascular disease [5]. Calcified arteries are particularly prone to dissection after angioplasty [6]. Venous calcification contributes to chronic venous insufficiency and venous hypertension [3]. In the calcified vessel, hydroxyapatite crystals have the potential to promote low-grade inflammation that favors loss of vascular integrity and plaque rupture, and contributes to thrombotic vascular occlusion and myocardial infarction [7].

Arterial calcification can be found in the intimal layer, the medial layer, or in association with the internal elastic lamina. Venous calcification is mainly restricted to the medial wall [3].

In atherogenesis, calcification occurs in the intima of the plaque and is predominantly associated with the plaque core [8]. Accordingly, hydroxyapatite crystals often collocate with lipid- and cell debris-rich areas showing

numerous foam cells and macrophages at the periphery. Plaque calcification is mainly driven by inflammatory mediators.

The second type of vascular calcification occurs in the medial layer. This type of mineralization—often referred to as Mönckeberg's sclerosis—can be widely spread through the vascular tree. In arteries, medial mineralization is associated with advanced age, diabetes, and renal failure. Calcification of the medial vein wall is often present in varicosis and chronic venous insufficiency. Another type of arterial wall calcification is the mineralization of the aortic valves, in native or graft tissue. Degenerative calcific aortic valve stenosis is the most common valvular lesion encountered in clinical cardiology [1, 2]. Hyperphosphatemia has been identified as a pathogenic factor causing medial calcification. A variant form of artery media calcification, concentrated at the internal elastic lamina, is associated with PP_i deficiency [9, 10].

Similarities in the mechanisms of vascular calcification and the pathways of bone formation have been reported. Different end points of mineralization are encountered in the vessel wall, namely, mineralized cartilage, precipitated hydroxyapatite crystals, and even mature bone tissue, including bone marrow. Likewise, different pathways of mineralization, that is, enchondral and desmal ossification or hydroxyapatite crystal precipitation, have been discussed for arterial calcification. However, whether all or which pathways are used in arterial wall mineralization is still a matter of debate.

This chapter focuses on recent developments in understanding the mechanisms of vascular calcification with special emphasis on the type of mineralization and on the impact of loss of inhibition.

3. Parallels in Arterial Calcification and Physiological Tissue Mineralization

Vascular smooth muscle cells (SMCs) as members of the mesenchymal cell differentiation tree, like osteoblasts and chondrocytes, are capable to form mineralizing, membrane-limited cell fragments (matrix vesicles) that provide a sheltered environment for the initiation of calcification [11, 12]. As shown by Boström *et al.* [13], cultured human aortic medial SMCs form nodules, that is, matrix vesicles that serve as foci of spontaneous calcification. The origin of calcifying SMCs (cSMCs) or osteoblast-type cells is still under discussion: Resident medial smooth muscle cells may differentiate into cSMCs. cSMCs may originate from immigrating osteoprogentitor cells, or resident pericytes may be activated *in situ* to differentiate into an osteoblast-like cell type [12]. Nevertheless, independent of their origin, cSMCs follow the critical sequence of expression of bone/cartilage-related proteins as in normal mineralizing bone [14]. As for bone mineralization, different

mechanisms of vascular matrix calcification exist [15]. cSMCs may produce an osteogenic matrix or provide matrix vesicles for the precipitation of hydroxyapatite crystals. The formation of an osteogenic matrix, which is directly mineralized as in desmal bone formation, is another possible mechanism. Furthermore, the formation of a primary cartilaginous matrix, which secondarily is remodeled to an ostoid and than mineralized as in enchondral bone formation, has been reported [16].

Recent studies in animal models and cell culture studies have demonstrated that (as in mineralizing bone) vascular calcification must be actively inhibited by physiological function of resident cells (SMCs and endothelial cells) and infiltrating cells (tissue macrophages). It has been reported that deficient expression of various inhibitors of calcification is sufficient to trigger the calcification process. Alternatively, vascular calcification can be the result of a systemic expression of procalcifying mediators, for example, inorganic phosphate (P_i). The end result includes expression of a number of cartilage or bone matrix proteins, which orchestrate a process resembling osteogenesis within the vessel wall [17, 18].

4. Inhibitors of Artery Calcification

Characterization of targeted or naturally occurring mutations of a variety of bone- and cartilage-associated genes in mice has identified 11 different inhibitors of vascular calcification *in vivo* (Table 1). One of these inhibitors is of specific importance with respect to our review, namely, the nucleotide pyrophosphatase/phosphodiesterase family member NPP1, since inborn deficiency of NPP1 is associated with calcification of arteries in mice and humans.

4.1. Nucleotide Pyrophosphatase/Phosphodiesterase 1 and ANK

NPP1 is a major generator of extracellular PP_i in cartilage and a variety of other tissues [10, 19]. PP_i potently inhibits hydroxyapatite crystal deposition and growth. The physiological role of NPP1-mediated PP_i generation on tissue calcification has come to light recently. First, an inactivating *enpp1* mutation was linked to the hypermineralizing phenotype of *ttw/ttw* mice [19]. The *ttw/ttw* mice develop mineralized articular cartilage and perispinal ligament calcification progressing to hyperostotic joints and spine ankylosis and show arterial calcifications in early life. A similar phenotype is found in *enpp1*-knockout mice. In these mice, hydroxyapatite crystals are found in the medial layer and at the level of the internal elastic lamina of the arteries. Ho *et al.* linked a similar mouse phenotype with hyperostosis and increased

TABLE 1
MOUSE MODELS OF VASCULAR CALCIFICATION

Gene symbol, human protein name, [OMIM entry no.]	Mouse model	Major phenotypic features	Proposed mode of arterial calcification	References
MGP, Matrix-Gla Protein [154870]	*mgp*$^{-/-}$	Arterial and cartilage calcification, tracheobronchial stenosis Human correlate: Keutel syndrome	Cartilaginous metaplasia and hydroxyapatite crystal deposition	[26–28]
ENPP1, Ectonucleotide pyrophosphatase/ phosphodiesterase 1 [173335]	*enpp1*$^{-/-}$	Articular cartilage calcification, hyperostosis, spine and peripheral joint fusion, arterial calcification Human correlate: Generalized arterial calcification of infancy	Cartilaginous matrix formation and hydroxyapatite crystal deposition	[21–23]
ANKH, Progressive ankylosis [605145]	*ank/ank*	Articular cartilage calcification, hyperostosis, spine and peripheral joint fusion, arterial calcification Human correlate: Ossification of the posterior ligament of the spine	Cartilaginous matrix formation and hydroxyapatite crystal deposition	[19–21]
OPG, Osteoprotegerin [602643]	*opg*$^{-/-}$	Osteoporosis, vascular calcification	Hydroxyapatite crystal deposition	[49–51]
Smad6/Madh6, SMA- and MAD-related protein 6 [602931]	*madh6*-mutant	Endocardial cushion defects, aortic ossification	Enchondral bone formation	[77]
FBN1, Fibrillin-1 [134797]	*mg*Δ/*mg*Δ, *mg*R/*mgR*	Aortic aneurysm, long bone overgrowth, medial arterial calcification Human correlate: Marfan syndrome	Hydroxyapatite crystal deposition	[62]

(*continues*)

TABLE 1 (*Continued*)

Gene symbol, human protein name, [OMIM entry no.]	Mouse model	Major phenotypic features	Proposed mode of arterial calcification	References
Car-2, Carbonic anhydrase-2 [259730]	*car-2*$^{-/-}$	Osteopetrosis, renal tubular acidosis, medial calcification of small arteries Human correlate: Carbonic anhydrase-2 deficiency	Hydroxyapatite crystal deposition	[43]
KL, *klotho* [604824]	*klotho*$^{-/-}$	Vascular calcification, rapid aging	Hydroxyapatite crystal deposition	[73]
AHSG, α2-HS-glycoprotein/fetuin [138680]	*ahsg*$^{-/-}$	Mild vascular calcification	Hydroxyapatite crystal deposition	[83, 86, 87]
OPN, Osteopontin [166490]	*opn*$^{-/-}$	Enhanced valve implant calcification	Hydroxyapatite crystal deposition	[36, 37]
ABCC6, ATP-binding cassette transporter subtype 6 [264800]	*dyscalc*	Myocardial necrosis and calcification, arterial calcification after freeze/thaw injury Human correlate: Pseudoxanthoma elasticum	Hydroxyapatite crystal deposition associated with mitochondria	[79–81]

calcification of cartilage and arteries to mutations of ANK, a protein which channels PP_i from the intracellular to the extracellular space [20].

In their study on the mechanism of arterial mineralization in *enpp1*-deficient mice, Terkeltaub and coworkers [21] showed that ectopic arterial calcification is mediated by an endochondral-like differentiation rather than simply by a dystrophic process. NPP1 and PP_i deficiencies stimulate the differentiation of SMCs to a calcifying/osteoblastic phenotype and thus promote arterial calcification. Treatment of mouse multipotential *enpp1*$^{-/-}$ bone marrow stromal cells with exogenous PP_i inhibited the formation of a cartilaginous matrix. *In situ* and *in vitro enpp1*$^{-/-}$ aortic cSMCs expressed a cartilage-specific gene pattern, that is, upregulated type II collagen and alkaline phosphatase (AP) expression, decreased expression of the calcification inhibitor osteopontin (OPN), and increased calcification. Moreover, this study provided first evidence that type X collagen, a collagen found in hypertrophic cartilage and potentially involved in cartilage mineralization, is expressed in the aorta during the process of calcification.

A similar phenotype is expressed by aortic SMCs from *ank/ank* mice *in vitro* and *in situ*. These SMCs are depleted of extracellular PP_i because of defective ANK transmembrane PP_i transport activity.

Taken together these data, one may assume that NPP1 and PP_i deficiencies induce the differentiation of SMCs to the so-called calcifying phenotype [21]. Analogous to enchondral bone formation, a cartilaginous matrix is produced which is secondarily mineralized by hydroxyapatite crystallization in the aorta of *ttw/ttw* or *ank/ank* mice. However, true bone structures as the expected end product of enchondral bone formation are missing.

In our group we correlated low systemic levels of PP_i with generalized arterial calcification of infancy (GACI), which is also known as idiopathic infantile arterial calcification (IIAC) [9, 10]. In patients with GACI, severe cardiovascular symptoms are usually apparent within the first month of life. Although survival to adulthood has been reported, most patients die because of heart failure within the first six months of life. In GACI, calcification of large- and medium-sized arteries associated with intimal hyperplasia occurs (Fig. 1).

In a boy of 5 years, we found a marked deficiency of PP_i-generating NPP1 activity in plasma and in an arterial biopsy sample [9, 10]. We recognized that arterial and peri-articular calcifications as well as PP_i and NPP1 deficiency are shared features of the GACI and the *ttw/ttw* phenotype. Therefore, we analyzed affected individuals with GACI from 11 unrelated kindred for mutations in *ENPP1*. In eight of these families, we found mutations of the *ENPP1* gene, either in homozygous or compound heterozygous state. We identified four different mutations resulting in premature termination codons and nine additional missense mutations distributed across the coding region from exon 3 to 25. For functional analysis, we transfected a pcDNA3

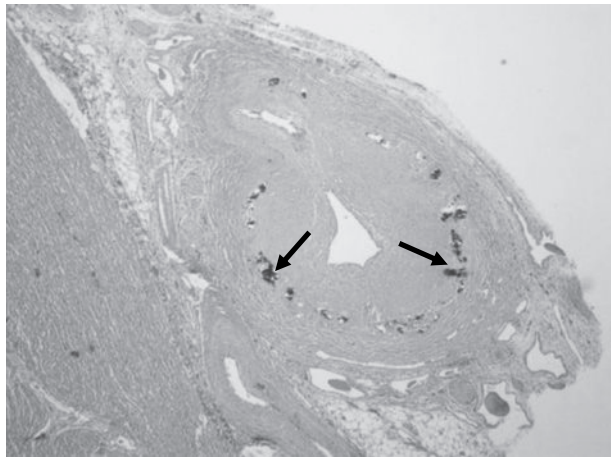

FIG. 1. Coronary artery calcification in human NPP1 deficiency: Hydroxyapatite crystal depositions (arrows) predominantly at the level of the internal elastic lamina in a patient with GACI, who died of myocardial infarction at the age of 4 weeks, note associated intimal hyperplasia. Von Kossa, original magnification ×100 (Courtesy of Galen Schauer, Department of Pathology, Children's Hospital and Clinics, Minneapolis, MN).

expression vector harboring the mutated full-length *ENPP1* coding sequence into SaO2 osteoblastic osteosarcoma cells and demonstrated complete loss of NPP1 activity in 9 of the 13 mutations tested [22]. These studies for the first time proved the linkage of genetically mediated dysfunction of NPP1 to arterial calcification and provided further evidence for abnormal PP_i metabolism to be an important regulatory factor for vascular and articular calcification. In a second study, we analyzed *ENPP1* in affected individuals of another 12 unrelated families. We identified 11 novel homozygous or compound heterozygous mutations in 10 of the 12 new families. The mutations (1 nonsense, 7 missense, 1 single amino acid deletion, and 2 frameshift mutations) were scattered over the whole coding region with a slightly more condensed distribution within the catalytic and nuclease-like domain as compared to the first survey [23].

Taken together our studies strongly emphasize the role of *ENPP1* mutations as the main cause of GACI (18 of 23 families).

Numakura *et al.* confirmed that an *ENPP1* mutation was also responsible for typical GACI in a Japanese patient. The homozygous Arg730Stop mutation was a novel nonsense mutation. Furthermore, they presented a late-onset phenotype patient without a mutation in the *ENPP1* gene [24]. Thus our data and theirs indicate that GACI is not a genetically homogeneous disorder, although *ENPP1* mutations are definitely the main cause of GACI.

Moreover, we found that despite a homogeneous genetic background and similar sonographic and radiographic features in early infancy, the cardiovascular phenotype of GACI can vary to a great extent within one family [25]. Of two Taiwanese siblings with identical genotype, one developed heart failure and severe hypertension, and died at the age of 6 weeks, while the other sibling is having an uncomplicated clinical course. Our data indicate that also in patients with the same *ENPP1* mutation additional factors may influence the clinical course.

4.2. Matrix-Gla Protein

Matrix-Gla protein (MGP)-deficient mice die 1–3 months after birth because a calcifying cartilage matrix associated with chondrocytes develops in the entire vascular tree [26]. Additionally, pathological calcification of various cartilages, including the growth plate, is leading to osteopenia, short stature, and fractures. Mutations of the *MGP* gene cause Keutel syndrome in humans, an autosomal recessive condition characterized by tracheobronchial stenosis, brachytelephalangism, cartilage calcifications [27], and, occasionally, arterial calcifications [28].

MGP inhibits matrix mineralization through suppression of bone morphogenetic protein 2 (BMP-2), a potent osteoinductive factor [29]. Recent *in vitro* studies suggest that MGP is a conditional enhancer or inhibitor of BMP-2-induced calcification, and that enhancement or inhibition depends on the specific BMP-2 levels relative to that of MGP [30]. MGP function depends on vitamin K-dependent γ-carboxylation of MGP glutamate residues. Price *et al.* treated rats with the vitamin K antagonist warfarin at doses that inhibited γ-carboxylation of MGP and found extensive vascular calcification and accumulation of undercarboxylated MGP [31]. These findings point to a potential mechanism linking warfarin to pathological soft tissue calcification. El-Maadawy *et al*. clearly demonstrated that in transgenic mice lacking MGP cartilage formation in the arterial media is caused by altered differentiation of resident vascular SMCs expressing chondrocyte markers [26].

4.3. Osteopontin

OPN is a multifunctional molecule regulating chronic inflammation and vascular mineralization [32]. OPN is a major noncollagenous matrix protein of bone and a constitutive component of normal elastic fibers in the skin and aorta [33, 34]. OPN, like PP_i, potently inhibits hydroxyapatite crystal deposition and calcification by SMCs *in vitro* [35]. OPN expression is typically upregulated at sites of ectopic soft tissue calcification, including artery calcification [34]. OPN is associated with mineralized deposits in humans in

atherosclerotic plaques [36]. Although unchallenged OPN-knockout mice are grossly normal, enhanced calcification of implanted glutaraldehyde-fixed aortic valve leaflets was demonstrated [37]. Using multimodality molecular imaging, Aikawa *et al.* [38] identified osteogenic activities colocalizing with various markers of bone mineralization, including OPN in the aortic valves of apolipoproteinE-deficient ($apoE^{-/-}$) mice. Since mineralization was not evident in the aortic valves (the aortic arch showed prominent calcification), they assumed an active process of ongoing mineralization from their data. From the expression of Notch 1, they deduced a mineralization process mediated via an osteoblast like SMC phenotype. However, whether the mineralization follows the enchondral pathway or another is still unclear.

In $apoE^{-/-}/OPN$-deficient mice vascular calcification is augmented. In these mice, calcified lesion areas in the intimal layer were about 2.5-fold increased in comparison to $apoE^{-/-}$ mice. With respect to the potential mechanism of mineralization, the histological data indicate hydroxyapatite crystal precipitation [39]. This mechanism has been proposed before, since OPN is a potent inhibitor of apatite growth by binding to the crystal surface *in vivo* [35, 40].

Significantly, PP_i deficiency promotes OPN deficiency, and correction of OPN deficiency corrects the hypercalcification state of NPP1-deficient mice, suggesting a synergistic effect of OPN and NPP1 on inhibiting hydroxyapatite deposition in arteries [41].

4.4. Carbonic Anhydrase-2

Carbonic anhydrase-2 provides protons and bicarbonate ions to the local microenvironment and allows for massive acid secretion that leads to degradation of organic matrix in bones [42]. Carbonic anhydrase-2–deficient mice show age-dependent medial calcification in arterioles and smaller arteries in numerous organs. The calcification apparently begins with hydroxyapatite crystal precipitation within the SMCs of the medial wall. The male genital tract revealed the most extensive arterial calcinosis and males were possibly more affected than females, indicating an effect of gender also on arterial wall mineralization [43]. In humans, the rare inherited disease of carbonic anhydrase-2 deficiency leads to osteoporosis, renal tubular acidosis and cerebral calcification [44]. Whether the acid secretion activity is a prerequisite to prevent vascular calcification is still unclear.

4.5. Osteoprotegerin

OPG, a recently identified member of the tumor necrosis factor (TNF) receptor superfamily, is a secreted factor that inhibits osteoclast differentiation and activation [45, 46]. OPG is also found in normal arteries and early

atherosclerotic lesions [47]. A T/C transition in the promoter region of the human OPG gene has recently been identified to significantly affect vascular morphology and function in healthy subjects [48].

OPG-knockout mice show severe bone loss and suffer from multiple osteoporotic fractures [49]. Surprisingly, the majority of the OPG-deficient mice develop severe medial calcifications of the renal arteries and the aorta. In these mice, aortic mineralization often is associated with dissection [50]. After induction of vascular calcification with a high-phosphate diet plus calcitriol treatment in OPG-knockout mice, needlelike calcium crystals were observed in the cytoplasm, SMCs, and in the extracellular space. Apoptotic or infiltrating cells were not detected. The mineralized areas show less elastic matrix and thinner SMC layers [51]. The vascular abnormalities and osteoporosis in OPG-knockout mice were completely abolished using an OPG transgene approach. However, intravenous injection of recombinant OPG could not reverse the medial calcification, suggesting local production is imperative for the inhibition of calcification [52]. Furthermore, the absence of OPG in *OPG/apoE* double knockout mice accelerates the mineralization of atherosclerotic plaques. In this context, an increase in lesion area was observed due to an increase in extracellular matrix components such as collagen, proteoglycans, and elastin. It is possible that the lack of OPG results in downregulation of matrix degrading enzymes [53]. Previous studies suggested that elastin can be a scaffold for deposition of hydroxyapatite; and with formation of the multiple layers of elastin, there may be an increased surface area to support calcification [54]. Previously it has been reported that chondrocyte-like cells are associated with the deposition of hydroxyapatite in atherosclerotic lesions of *apoE*-knockout mice [55]. In *OPG/apoE* double knockout mice, Bennett *et al.* found chondrocyte-like cells as well. However, there was a decrease in total cellularity in the lesions, including the number of chondrocyte-like cells. Thus, the absence of OPG does not appear to stimulate chondrocyte metaplasia [53].

In human abdominal aortic aneurysms (AAA), OPG expression was upregulated in less-calcified areas and downregulated in severely calcified areas. This might reflect the counterregulatory mechanism of OPG in calcified areas [56]. OPG serum levels have been associated with cardiovascular morbidity and mortality in elderly women [57]. In humans, OPG serum levels may not only reflect the disposition for cardiovascular diseases but also for chronic renal failure and diabetes [58–60]. OPG may be a key factor illuminating the underlying mechanism of the well-known paradoxical association of arterial calcification and osteoporotic loss of bone mass in elderly humans. Taken together, these studies indicate that serum OPG levels play a critical role in clinical conditions that favor atherosclerosis, vessel calcification, or vascular dysfunction, and they suggest that OPG may have an important role in the cause or progression of vascular calcification.

4.6. Fibrillin-1, Elastin Disintegration, and Aneurysm

Fibrillin-1 mutations result in the pleiotropic manifestations of Marfan syndrome [61], which is associated with hypermobility, aortic aneurysm formation, and calcification and long bone overgrowth. Fibrillin-1 appears to be essential for homeostasis of established elastic fibers and for cell adhesion involved in remodeling the matrix [62]. Mice deficient in fibrillin-1 revealed a predictable sequence of abnormalities including elastic fiber calcification, excessive deposition of other extracellular matrix molecules, elastolysis, and intimal hyperplasia. Bunton *et al.* showed that in elastic arteries of patients with Marfan syndrome, these changes are related to an SMC phenotype change (potentially to the so-called cSMC phenotype) [63]. This results in an abnormal elastic matrix, which serves a focus for hydroxyapatite crystallization. In the rat model of $CaCl_2$-mediated aortic injury, elastic lamellae stabilization through periadventitial administration of pentagalloyl glucose did not interfere with calcification [64]. Taken together, these data indicate that both loss of cell adhesion sites and elastin disintegration is necessary for elastic lamellae calcification. However, even without fibrillin-1 deficiency patients with aortic aneurysms (and thus with degraded elastin fibers but intact cell adhesion sites) often show arterial wall mineralization. In patients with small AAA, the annual growth rate is lower in patients with a higher degree of calcification (indicating an influence of mineralization of vessel wall stabilization) [65].

Onset and progression of elastin calcification has been related to elastin degradation [66]. Matrix metalloproteinase (MMP) activation, in particular that of the elastolytic MMPs 2, 3, and 9, was found to be associated with increased elastin calcification. Vyavahare et al. demonstrated that both gelatinases (MMP-2 and MMP-9) are involved in elastin-oriented calcification [67]. Coronary artery calcification has been related to a functional polymorphism of the elastin degrading MMP-3 [68]. In an autopsy series of coronary arteries of 300 middle-aged white Finnish men, the subjects with high MMP-3 promoter activity had larger calcified lesion areas than those subjects within the low-activity group. In reverse, inhibition of MMPs significantly reduces elastin calcification. Administration of aluminum ions (known inhibitors of elastin degradation) reduced CaCl2-induced chronic degeneration and calcification of elastic fibers in a rat model [69]. Accordingly, MMP-knockout mice were resistant to CaCl2-mediated aortic injury and did not develop elastin degeneration and calcification [69]. Application of synthetic inhibitors of MMPs—such as GM1001 or BB1101—suppressed MMP activity, prevented MMP-mediated elastolysis, and thus inhibited calcification of elastin in subepidermally implanted purified elastin and in vitamin D3 induced aortic calcification in rats [67, 70].

In atherosclerosis, additional elastic fiber dysfunctions, that is, downregulation of elastin, fibrillin, and lysyl oxidase, are directly related to SMC calcification [71]. In this respect, it has been shown that increased tropoelastin inhibits vascular calcification in cultured SMCs via the interaction between tropoelastin- and elastin-binding protein [72].

From these data one may assume that an intact elastic system and also subcomponents of the elastic lamellae inhibit vascular mineralization, that is, hydroxyapatite crystal precipitation.

4.7. Klotho

Mutations in the mouse *klotho* gene encoding β-glucosidase have been demonstrated to cause a phenotype resembling human aging, which also includes medial arterial calcification [73]. A twofold increase in serum P_i caused by elevation of renal type IIa Na/P_i cotransporter activity appears to be the main pathogenetic factor and phosphate retention rescues the phenotype [74]. Interestingly, *klotho*-deficient mice also develop intimal thickening [73]. The calcified lesions appear in sheets within the arterial media, therefore resembling Mönckeberg's media sclerosis. The expression of cartilage-specific markers has not been studied in the arteries of *kl/kl* mice. The *klotho* allele status seems to affect the risk of human coronary artery disease, since the *KL-VS* allele, harboring two amino acid substitutions in complete linkage disequilibrium, was associated with occult coronary artery disease in a cross-sectional association study by Arking *et al.* [75].

4.8. Smad6

A most striking vascular phenotype is present in mice lacking Madh6, the mouse homologue for Smad6. Smad6 plays a pivotal role in negative regulation of transforming growth factor-β (TGF-β) family signaling as a feedback molecule as well as a mediator of cross talk with other pathways [76]. Specifically, Smad6 has been shown to effectively inhibit BMP signaling through interaction of BMP type I receptors [77]. Knockout mice for the Smad6 homologue develop cartilaginous metaplasia and trabeculated bone in the aortic wall [78]. Thus, in these mice, the complete process of enchondral bone formation is recapitulated within the arterial wall.

4.9. ABCC6 (ATP-Binding Cassette Transporter Subtype 6)

In 1996, Ivandic *et al.* identified a locus on chromosome 7 determining myocardial cell necrosis and calcification (dystrophic cardiac calcinosis) in certain inbred strains of mice [79]. Dystrophic cardiac calcinosis occurred

in these mice as response of myocardial tissue to injury. Ultrafine-mapping of the Dyscalc locus on chromosome 7 later revealed an 80-kb segment harboring the responsible gene [80], which was recently identified as *Abcc6* (ATP-binding cassette transporter subtype 6) [81]. Mutations in *Abcc6* have been known to be associated with Pseudoxanthoma elasticum (PXE) [82]. In PXE, the skin of the neck, axilla, and other flexural areas becomes lax, redundant, and relatively inelastic. In the eye, funduscopic examination shows "angioid streaks" in the Bruch's membrane. Vascular findings include occluded arterial vessels, calcification, and gastrointestinal hemorrhage. Arteries from *dyscalc* mice harboring *Abcc6* mutations, which were subjected to freeze/thaw injury, also show patchy dystrophic calcification. These calcifications were demonstrated to originate from mitochondria of apoptotic vascular SMCs (L. Doehring, personal communication).

4.10. Fetuin-A (α-Heremans Schmid Glycoprotein)

Fetuin-A is a major systemic inhibitor of calcification, accounting for approximately 50% of the precipitation inhibitory capacity of serum [83, 84]. *In vitro*, fetuin-A is a highly potent inhibitor of hydroxyapatite formation and precipitation by forming a soluble "calciprotein" peptide. In addition, fetuin-A promotes endocytosis and serves as an opsonin to promote phagocytosis, thus favoring the removal of insoluble calcium remnants [85]. Furthermore, fetuin-A has been shown to regulate several of the key cellular events that lead to vascular SMC calcification, including apoptosis, vesicle calcification, and phagocytosis [86]. In fetuin-A-deficient mice, the degree of mineralization depends on the genetic background. On a DBA2 background, fetuin-A-deficient mice spontaneously develop severe calcifications in small blood vessels and other soft tissues [87]. A milder calcified phenotype is expressed on a C57BL/6 background only after vitamin D challenge or after feeding mineral-rich diet. Generally, the calcifications were extracellular and most prominent in organs involved in the secretion or transport of mineral-rich fluids or in the generation of local pH changes. Further electron microscope analysis revealed the presence of calcium phosphate deposits in foam cell-like macrophages. The extensive ectopic calcifications in fetuin-A-deficient DBA2 mice lead to arterial hypertension and renal failure [88]. In patients with chronic renal failure, low fetuin-A serum levels are associated with vascular and ectopic calcification leading to increased cardiovascular mortality in hemodialysis patients [89]. Moe *et al*. [90] demonstrated fetuin-A immunostaining associated with areas of calcification in arteries from five patients with chronic kidney disease. Furthermore, decreased serum fetuin-A levels were observed in PXE patients, characterized by progressive calcification of abnormal and fragmented elastic fibers [91]. Serum fetuin-A is

increased in patients with peripheral vascular disease and increased carotid arterial stiffness [92, 93]. Polymorphisms in the fetuin-A gene lead to decreased serum fetuin-A levels and increased serum phosphate levels, suggesting that genetic variants resulting in changes in protein function may predispose to calcification [94, 95]. In individuals with type 2 diabetes mellitus, sequence variants in the fetuin-A gene affect the extent of coronary artery calcified plaque [96]. Since a human syndrome characterized by complete deficiency of fetuin-A has not been described yet, a complete lack of fetuin-A may be lethal.

5. Promoters of Arterial Calcification

In tissues not meant to calcify, pathological calcification can be both the consequence of deficiencies of calcification inhibitors and a response to upregulated action of promoters of calcification (Fig. 2). Calcification in atherosclerotic vessels exemplifies this model, as several mineralization-regulating proteins are upregulated in calcifying atherosclerotic lesions, including BMP-2, BMP-4, and the stereotypic osteoblastic proteins

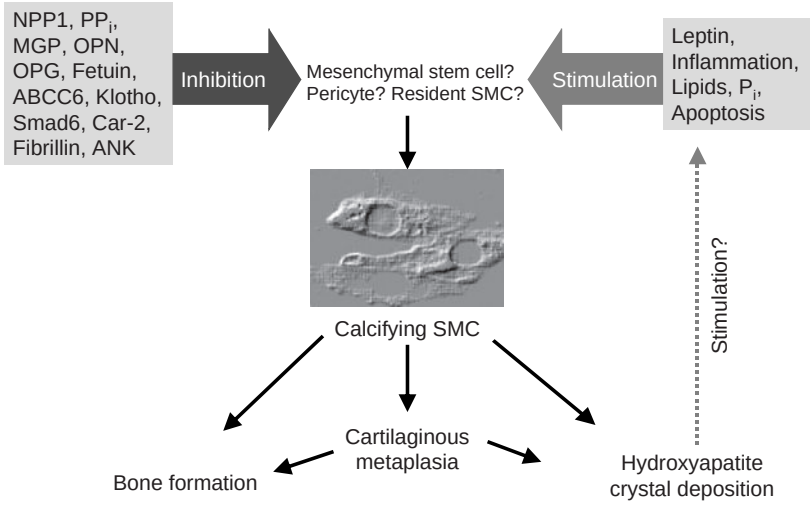

FIG. 2. Mechanisms of arterial calcification. Deficiency of inhibitors and/or gain of promoters of calcification induce the transdifferentiation of precursor cells to the cSMC phenotype. This triggers an orchestrated process of matrix mineralization, which can lead to different end-points. Hydroxyapatite crystal deposition again stimulates the inflammatory process leading to a vicious circle.

osteocalcin and osteonectin [13, 34, 47, 97]. Within recent years, a number of specific promoters of arterial calcification have been identified, which counteract the inhibitors addressed above.

5.1. Inorganic Phosphate

SMCs in the process of mineralization *in vitro* undergo a phenotypic transition toward an osteogenic phenotype [98]. P_i drives expression of several osteoblastic genes in cultured SMCs [99]. Concordantly, osteoblastic differentiation of SMCs likely mediates pathological medial calcification [including coronary artery calcification and calcific uremic arteriolopathy (CUA) in hyperphosphatemia such as in ESRD] [100, 101]. P_i uptake by SMCs is mediated by a sodium-dependent phosphate cotransporter identified as Pit-1 (also termed Glvr-1) implicated in osteoblast differentiation and calcification. [99]. Significantly, Pit-1 is regulated by a variety of growth factors [101, 102] and by epinephrine [103]. These observations have suggested that modulation of cellular "P_i hunger" might regulate pathological calcification.

Renal proximal tubular reabsorption is a key element for overall P_i homeostasis. Interestingly, *klotho* mutations (Table 1) lead to an elevation of renal type IIa Na/P_i cotransporter activity and thereby increase phosphate reabsorption in the kidney leading to vascular calcifications [74]. Also, according to a recent study, arterial calcification was more extensive in ESRD patients with biopsy-proven low bone activity and adynamic bone. Therefore, the well-known association between hyperparathyroidism and arterial calcification is more likely related to the high-bone-turnover-associated release of calcium and phosphate from bone than to the direct action of parathyroid hormone [104]. On the other hand, increased P_i clearance as seen in hypophosphatemic rickets is able to compensate typical phenotypic features of infantile arterial calcification [22].

Low serum phosphate levels should therefore be maintained in patients at risk for ectopic calcification. This is already taken into account in the most recent guidelines for phosphate management in hemodialysis patients [105].

5.2. Potential Effects of Leptin

Calcification in SMCs also is promoted by the satiety factor leptin, which enhances mineralization by cSMCs *in vitro*, in part by increasing AP activity [106]. A leptin receptor has been identified on cSMCs, as well as on medial and adventitial cells in the mouse artery wall [106]. Leptin-deficient and leptin receptor-deficient mice are protected from arterial thrombosis and neointimal hyperplasia in response to arterial wall injury. Several clinical

studies have demonstrated that high leptin levels predict acute cardiovascular events and cerebral stroke independently of traditional risk factors. In addition, plasma leptin correlates with markers of subclinical atherosclerosis, such as carotid artery intima–media thickness and coronary artery calcifications. Inhibition of leptin signaling may therefore be a promising strategy to slow the progression of atherosclerosis in hyperleptinemic obese subjects [107].

5.3. Promineralizing Effects of Apoptosis

Apoptotic processes predominantly adjacent to calcified areas have been detected in the media in Mönckeberg's sclerosis and the neointima in atherosclerosis [108]. Recently, it has been shown that similar structures to matrix vesicles, derived from apoptotic SMC, have been identified in human calcified arteries. These "apoptotic bodies" have the capacity to concentrate both calcium and phosphate to generate hydroxyapatite, initiating calcification. Inhibition of apoptosis appears to reduce calcification *in vitro*. Similarly enhancement of apoptosis increased calcification [109]. Phosphatidylserine is located on the outer membrane surface of apoptotic bodies and therefore faces the extracellular milieu [110]. Phosphatidylserine has been shown to bind calcium, and the mineral phase in matrix vesicles is associated with phosphatidylserine [111]. Therefore, it is plausible that apoptotic bodies accumulate calcium and P_i on their outer surface through their external phosphatidyserine [112]. Apoptotic processes in arteries may be induced by orthotopic bone formation protein BMP-2 [113], P_i [114], and the cytokine TNF-related apoptosis-inducing ligand (TRAIL) [108]. Of interest, OPG has been described to bind TRAIL and thereby to inhibit its cytotoxic capacity in an *in vitro* model [115]. Increased OPG production in apoptotic areas may indicate a counterregulatory mechanism to limit apoptosis and thereby the initiation of calcification [108]. Phosphate-induced SMC calcification may be inhibited by statins by preventing apoptosis via restoration of the Gas6–Axl pathway [113]. In most situations, cell debris is rapidly cleared by phagocytic cells [116]. However, clearance of SMC debris may be inhibited, leading to accumulation of apoptotic bodies [117, 118], which proceed to calcify.

5.4. Role of Lipids

Clinical studies on arterial calcification have consistently shown that high levels of serum low-density lipoprotein (LDL) cholesterol and low levels of serum high-density lipoprotein (HDL) cholesterol are directly related to the prevalence and extent of coronary artery calcification [119–121]. Oxidized lipids generated by activated macrophages, endothelial cells, and SMCs in the course of atherogenesis, also promote calcification partly by

compromising cell viability and by direct modulation of hydroxyapatite deposition in the matrix [122].

Transgenic mice such as the *apoE*$^{-/-}$ and LDL-receptor-deficient (*ldlr*$^{-/-}$) mice exhibit hypercholesterolemia and develop complex atherosclerotic lesions similar to those seen in humans [123, 124]. Rattazzi *et al*. characterized advanced atherosclerotic lesions in the innominate arteries of chow-fed *apoE*$^{-/-}$ mice and focused on plaque calcification. They observed that by 75 weeks of age there was a 100% frequency of calcification. Furthermore, it appeared that chondrocyte-like cells were responsible for depositing hydroxyapatite crystal formation through a process that recapitulates the cellular and temporal aspects of endochondral ossification, including a developing bonelike expression pattern, the presence of active AP in chondrocyte-like cells within and adjacent to the areas of calcification, and the presence of matrix vesicles derived from chondrocyte-like cells [55]. In contrast, chow-fed *ldlr*$^{-/-}$ mice did not show any calcification in arteries. However, *ldlr*$^{-/-}$ mice crossed with ApolipoproteinB-100-only mice developed aortic stenosis, valvular heart disease, oxidant stress, and calcification in aortic valve tissue on normal chow diet [125]. On the other hand, aortic mineralization was consistently observed in *ldlr*$^{-/-}$ mice on a carbohydrate diet without cholesterol and in animals on a high-fat diet containing 0.15% cholesterol, independent of atheroma formation. These mice showed expression of *Msx2* and *Msx1* (transcription factors controlling neurectodermal osteoblast differentiation) and osteopontin. These data suggest that diet-induced calcification was caused by an active process, via initiation of an osteoblast transcriptional regulatory program [126].

In a European American diabetic population, several common variants of genes involved in lipid metabolism (*PON1, PON2, LPL*) appeared to contribute to arterial calcification [127].

In vitro, HDL may regulate calcification of so-called cSMCs. cSMCs treated with HDL had significantly lower AP activity, an established marker of osteoblastic differentiation in osteoprogenitor cells, including cSMCs. HDL also blocked matrix calcification in cSMC cultures, suggesting that HDL regulates early and late events in osteogenic differentiation. Furthermore, HDL blocked the calcification induced by inflammatory cytokines interleukin (IL)-1β and IL-6 as well as that induced by minimally oxidized LDL. In contrast, after oxidation, HDL not only lost its antiosteogenic effect but also induced calcification of cultured cSMCs [128]. HDL may prevent calcification of vascular cells and thus prevent calcification-induced vascular complications that would impair the proper functioning of the vessel wall.

Lipid-lowering therapy by statins decreases progression of coronary artery and aortic valve calcification [129, 130]. Hypercholesterolemic aortic valve calcification is attenuated by atorvastatin and is mediated in part by the

Lrp5–beta-catenin pathway [130]. As shown by Kizu *et al.* [129], in vascular SMC statins interfere with the induction of AP by inflammatory mediators, such as interferon gamma, vitamin –D3, TNFα, and oncostatin M. This inhibitory effect of statins on vascular mineralization is mediated via Rho kinase.

5.5. Low-Grade Inflammation Promotes Arterial Calcification

Atherosclerosis and valvular disease [2, 131], varicosis [3], ESRD, and diabetes mellitus [132], all etiologies associated with vascular mineralization are recognized as inflammatory states. Evidence steadily is mounting that inflammatory processes play an important role in vascular calcification. Vascular calcification in general is associated with infiltration of inflammatory cells and expression of inflammatory mediators.

In a rat model of elastocalcinotic arteriosclerosis, media calcification was associated with macrophage/monocyte infiltration and induction of TNFα and IL-1β. In this animal model, aortic wall calcification was blunted by anti-inflammatory treatment with Pioglitazone [133].

In Takayasu arteritis (TA), a chronic inflammatory disorder affecting the aorta and its branches, vascular calcification has been described in up to 54% of the cases. In TA, severe arterial calcification is associated with low bone mineral density and low expression of the receptor activator of nuclear factor-kappaB ligand (RANKL), reinforcing the link between osteoporosis and vascular calcification [134, 135].

Atherosclerosis is conceptualized as a chronic inflammatory process in which numerous inflammatory cell types and cytokines are known to participate [136]. The groups of Doherty and Demer and coworkers have reviewed atherosclerotic lesion calcification in depth [122, 137]. As reported for other vascular mineralization types, intimal calcification in atherosclerotic plaques is an organized process carried out by ectopic osteoblastic and chondrocytic cells and regulated by osteoclastic cells in the inflammatory lesions [137]. Infiltrating inflammatory cell types are critical in the pathogenesis of atherosclerosis and macrophages colocalize with calcific deposits in atherosclerotic plaques [136, 138, 139]. Particularly, the cytokines secreted by inflammatory cell types are of importance for vascular mineralization processes [140, 141]. In a coculture model, in the presence of interferon gamma and vitamin D, macrophages stimulated the expression of AP and under addition of beta-glycerophosphate matrix mineralization by SMCs [142]. TNFα and oncostatin M, only when secreted together by macrophages, were identified as inducers of AP, which plays a fundamental role in bone mineralization [143]. TNFα is secreted by macrophages in response to certain atherogenic factors, including oxidized LDL [144], and it promotes osteoblastic gene expression

and calcification in cultured bovine SMCs [18]. IL-4—secreted by lymphocytes and linked to atherosclerosis—exerts differential osteogenic effects on vascular SMCs [145]. While short-term exposure enhances OPG production, long-term exposure causes Cbfa1-dependent osteogenic transdifferentiation of SMCs and decreased OPG expression [145]. Insulin-like growth factor-1 (IGF-1), one of the paracrine regulators of osteoblasts, is also present in atherosclerotic lesions. IGF-1 inhibits the spontaneous transdifferentiation of SMCs to the calcifying phenotype and mineralization [141].

On the other hand, macrophages may promote a vicious circle of inflammation and calcification in the vessel wall by ingesting intimal calcific deposits and producing procalcifying agents such as TNFα. TNFα secretion was inversely correlated to hydroxyapatite particle size indicating that microscopic calcific deposits in early stages of atherosclerosis may pose a greater inflammatory risk to the plaque than the macroscopically visible deposits in more advanced lesions [146].

In their study on severely calcified and stenotic human aortic valves, Anger *et al.* [147] provided evidence that atherosclerotic inflammation is a trigger for sclerosis in aortic valvular disease. Atherosclerotic target genes, such as TGFβ and vascular adhesion protein-1, were upregulated in mineralized aortic valves. Previous statin therapy as part of an anti-inflammatory treatment strategy could only partially reverse this effect.

Inflammation and loss of extracellular matrix integrity (elastolysis) are hallmarks of abdominal aortic aneurism (AAA). AAA is commonly associated with atherosclerosis and calcification. In AAA expression of TRAIL and its inhibitor OPG correlated with the extent of calcification [56].

In patients with end-stage renal failure, increased CRP levels are associated with the presence of vascular calcification (atheromatous and medial forms) [148].

TNFα, the OPG–RANKL–RANK system and colony-stimulating factor 1 have also been implicated in osteoclast formation and function. These cytokines are also expressed in atherosclerotic plaques, consistent with the concept of the presence of osteoclast-like cells in arteries, which might derive from resident macrophages of the plaque [136].

6. Implications of Basic Research on Clinical Therapy

Studies on knockout mice have led to the identification of a substantial number of inhibitors of vascular calcification. A few of them have already proven their potential to be translated into therapeutic targets for future clinical trials. Recently, for example, vascular calcification as a known side effect of coumadin therapy has been attributed to underdecarboxylation of

MGP [31]. This effect was shown to be antagonized in rats by high intake of vitamin K [149]. Two independent studies have demonstrated that subjects on long-term anti-coagulation have more arterial and heart valve calcification than an age- and sex-matched control population [150, 151]. Future clinical studies should therefore address the influence of vitamin K on MGP carboxylation and vascular calcification in the setting of long-term warfarin therapy.

Vascular calcification is a major determinant of morbidity and mortality in ESRD. In our view, two major candidates are of utmost importance for future clinical trials in this respect, namely, P_i and PP_i. Dietary restriction and conventional dialysis are ineffective in controlling hyperphosphatemia in ESRD patients; thus, the majority of dialysis patients require phosphate binders. Until recently, the predominant binders in use have been calcium acetate and calcium carbonate. However, these agents have been linked to arterial calcification [152]. In a most recent study, treatment with sevelamer, an anion-exchange resin, which sequesters phosphate within the gastrointestinal tract and therefore prevents its resorption and enhances fetal excretion [153], was associated with lower overall mortality [154]. Deficiency of extracellular PP_i has been identified as the cause of arterial calcification in infantile arterial calcification [9, 10]. Similarly, in uremic patients, low plasma pyrophosphate levels were demonstrated [155]. When administered orally, pyrophosphate is subjected to rapid degradation, therefore bisphosphonates, which are structural analogues resistant to enzymatic hydrolysis, have been used. In high doses, these compounds physicochemically prevent mineralization by inhibiting the formation and aggregation of calcium phosphate crystals and blocking the transformation of amorphous calcium phosphate to hydroxyapatite [156]. Tamura *et al.* added to the growing body of evidence that bisphosphonates inhibit arterial calcification *in vivo* by showing a significant effect on aortic calcification in uremic rats [157]. A study in which bisphosphonates were administered to hemodialysis patients already generated hopeful results [158]. However, in apoE-knockout mice, bisphosphonate treatment was associated with a progression of atherosclerosis [159]. These conflicting results should lead to further clinical studies, since there is a substantial unmet medical need for effective drugs to prevent and treat arterial calcification.

7. Conclusion

Calcification in arteries can result from a deficiency of specific inhibitors. Targeted deletions of certain genes in mice models have identified NPP1, fetuin-A, Klotho, and a variety of other proteins, expression of which in the

arterial wall or in the circulation is pertinent for the integrity of the arterial extracellular matrix and for the inhibition of hydroxyapatite crystal precipitation. Likewise, a gain of promoters, such as increased low-grade inflammation and apoptosis, contributes to a transition to the cSMC phenotype. This leads to an orchestrated process of matrix calcification, which can occur at different sites of the arterial wall (intima and media), employing the instruments of bone formation, but reaching different end points, namely, pure deposition of hydroxyapatite crystals, cartilaginous metaplasia or complete bone formation (Fig. 2). While the etiology of these processes has come to light in recent years, the underlying mechanisms are still not fully elucidated. MGP, P_i, and PP_i have already proven their potential as valuable therapeutic targets, and further studies are warranted to optimize prevention and treatment of arterial calcification in patients at risk.

Acknowledgments

The authors are supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 492).

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ATHEROGENIC LIPOPROTEIN SUBPROFILING

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1. Abstract

Elevated levels of low-density lipoprotein (LDL) cholesterol are a major risk factor for coronary heart disease. However, a significant number of coronary events occur in individuals with "normal" serum LDL cholesterol levels. It has been proposed that coronary heart disease (CHD) risk stratification may be improved by evaluating LDL particle size and number and lipoprotein (a) [Lp(a)] levels in addition to LDL-associated cholesterol levels. This chapter includes a brief review of lipoprotein biology and presents an overview of the association between novel lipoprotein markers and CHD risk. We also discuss methodologies currently available for atherogenic lipid subprofiling.

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0065-2423/08 $35.00
DOI: 10.1016/S0065-2423(08)00408-3

2. Introduction

CHD is a manifestation of atherosclerotic vascular disease that is characterized by lipid-laden plaque accumulation within arterial walls accompanied by varying degrees of inflammation and fibrosis [1]. Dyslipidemia is a major risk factor for the initiation and progression of atherosclerotic lesions, and evaluation of dyslipidemia is an essential component of assessing CHD risk. In 1985, the National Heart, Lung, and Blood Institute launched the National Cholesterol Education Program Adult Treatment Panel (ATP) guidelines to assist clinicians in management of dyslipidemia. These guidelines recognized elevated total cholesterol and low-density lipoprotein cholesterol (LDL-C) as central lipid-related CHD risk factors [2]. However, a significant number of CHD-related events may occur in individuals who have serum LDL-C levels within "normal" limits, and there is intense interest in lipoprotein subprofiling to improve risk stratification. For instance, measurement of LDL particle size and particle number has been proposed as an alternative to simply assessing serum LDL-C levels [3, 4]. In this chapter, we provide a brief summary of lipoprotein biology and metabolism, review the epidemiological evidence for the association of novel lipoprotein parameters with CHD risk, and describe the methodologies available for atherogenic lipoprotein subprofiling.

3. Lipoprotein Biology

3.1. Lipoprotein Structure

Lipoproteins are spherical particles composed of a lipid monolayer surrounding a hydrophobic core that contains neutral triglycerides (TG) and cholesterol esters (CE; Fig. 1) [5]. The monolayer is composed of amphipathic phospholipids (PLs), proteins, and free cholesterol, features that confer water solubility to the lipoprotein particles. Apoproteins (Apos), the associated integral and peripheral proteins, provide structural stability and serve as receptor ligands and cofactors for enzymes involved in lipid metabolism. Each Apo, with the exception of ApoB, can be exchanged between lipoprotein particles [6], and this attribute modulates lipoprotein particle metabolism and clearance.

3.2. Classification of Lipoproteins

Various schemes are used to classify lipoprotein particles. Classification based on the structural Apo component divides lipoproteins into two families: ApoB-containing particles and ApoA-containing particles. Further

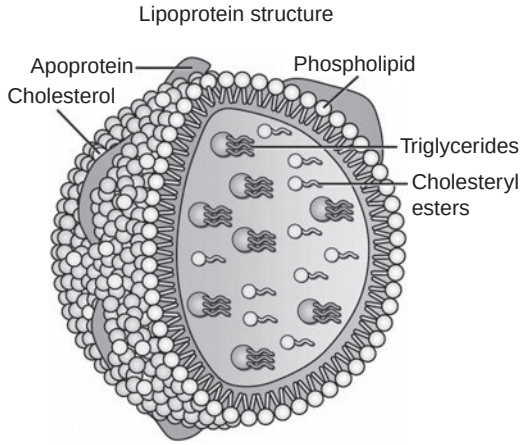

FIG. 1. The structure of a serum lipoprotein. A lipoprotein particle typically comprises a hydrophilic shell composed of phospholipids, apoproteins, and free cholesterol surrounding a hydrophobic core that contains triglycerides and cholesterol esters.

subclassifications are based on functional Apo components [7]. Lipoproteins belonging to the ApoB family are considered to be atherogenic and include chylomicrons (CM), very low density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), and LDLs. Each particle contains one molecule of ApoB, either the full-length ApoB-100 or the splice variant ApoB-48. ApoB-100 accounts for approximately 30%, 60%, and 95% of the protein found in VLDL, IDL, and LDL, respectively, whereas ApoB-48 is present only in CM [5]. In addition, each lipoprotein particle contains variable quantities of ApoC and ApoE.

More commonly, lipoproteins are classified into five main groups based on density. In order of decreasing size and increasing density these are CM, VLDL, IDL, and LDL (Table 1) [8, 9]. Lp(a), an LDL variant characterized by the covalent attachment of an Apo(a) moiety to ApoB-100, represents a sixth group with broad variability in size and density (Table 1) [9–11]. The ApoB family includes polydisperse lipoproteins that are heterogeneous in size, density, and lipid/protein ratio. Lipoprotein density is a function of the lipid/protein ratio, and low-density particles have a higher lipid/protein ratio than do more dense particles. Lipoprotein particle size is also a function of the nuclear lipid content, with larger particles containing increased lipid mass. For convenience, the terminology "large" and "small" particles will be used throughout this chapter to discuss lipoprotein subclasses. In regard to VLDL, at least two biologically relevant subclasses exist: large VLDL that contains a larger quantity of TG and small VLDL that is enriched with CE [12]. Predominant IDL

TABLE 1
PROPERTIES OF SERUM LIPOPROTEIN CLASSES[a]

Lipoprotein	Size (nm)	Density (g/ml)	Principal lipids	Structural apoprotein
Chylomicron	75–1200	<0.94	Dietary TG	ApoB-48
VLDL	30–80	0.94–1.006	Endogenous TG	ApoB-100
IDL	25–35	1.006–1.019	TG and CE	ApoB-100
LDL	18–25	1.019–1.063	CE	ApoB-100
HDL	5–12	1.063–1.125	CE	ApoA-I
Lp(a)	25–30	1.040–1.090	CE	ApoB-100

[a] Adapted from Cham *et al.* [12] and Olsen *et al.* [13]

VLDL = Very low density lipoproteins; IDL = intermediate-density lipoproteins; LDL = low-density lipoproteins; HDL = high-density lipoproteins; Lp(a) = lipoprotein (a); TG = triglycerides; CE = cholesterol esters; ApoB = apoprotein B; ApoA = apoprotein A.

subclasses include large IDL and small IDL. Notably, small IDL appears to form a continuum with small VLDL particles [12]. Two discrete subclasses of LDL have been identified: large, buoyant LDL and small, dense LDL (sd-LDL) [13]. Lp(a) ranges in size between 200 and 800 Da, a characteristic determined by the number of Kringle IV_2 repeats [10, 11].

3.3. METABOLIC MODELS OF LIPOPROTEIN SUBCLASS FORMATION

The predominant function of lipoproteins is to transport TG and cholesterol to peripheral tissues where TG are either used for energy or storage and cholesterol is used for biosynthesis of cell membranes and steroid hormones [14, 15]. Excess cholesterol in the periphery is removed by HDL through the process of reverse cholesterol transport (RCT) and delivered to either the liver or steroidogenic tissues or transferred to ApoB-100 lipoproteins [16–19].

ApoB lipoprotein subclass distribution is dependent upon the channeling of lipid substrates (cholesterol and TG) toward formation of particles varying in size, composition, and biological significance. Through the use of metabolic tracer studies, Packard *et al.* [14, 20, 21] have characterized the formation and metabolism of ApoB-100 lipoprotein subclasses and described two metabolic models that explain variations in lipoprotein subclass distribution. Differential metabolism and clearance of ApoB-100 subclasses create particles that differ in atherogenicity.

3.3.1. *Small VLDL Particles*

Small VLDL particle synthesis increases when cholesterol availability is in excess of TG availability [5]. Small VLDL derived from hepatic production is either cleared immediately through LDL-receptor (LDL-R) ligation or

delipidated by both lipoprotein lipase (LpL) and hepatic lipase (HL) [15, 22, 23]. Hydrolysis of TG in small VLDL particle cores generates IDL of which approximately 50% are removed from circulation through LDL-R ligation. The remainder is further delipidated by HL to produce large LDL particles readily cleared by LDL-R [14, 15, 21, 24]. Notably, the bulk of circulating LDL-C is generated through this delipidation cascade. This efficient delipidation channel limits reductions in small HDL particles catalyzed by cholesterol ester transfer protein (CETP) and HL metabolism.

3.3.2. *Large VLDL Particles*

Excessive levels of TG (as a result of obesity, untreated diabetes, diets rich in refined carbohydrates and alcohol consumption) cause increases in hepatic synthesis of large VLDL particles [5]. Following secretion, approximately 50% of large VLDL is cleared through LDL-R ligation whereas the remainder undergoes LpL-mediated TG hydrolysis to form IDL and LDL [14, 20, 21]. Lower-density lipoprotein species generated through this metabolic route appear relatively resistant to further LpL-mediated delipidation and remain in circulation for extended periods, enabling lipoprotein particle remodeling [14, 20, 21]. As large VLDL particles and metabolites accumulate, CETP-mediated lipid transfer occurs between ApoB-100 and HDL particles. This results in TG enrichment of small HDL particles, creating large HDL particles and CE enrichment of ApoB-100 particles. Larger HDL particles are substrates for HL, and delipidation creates readily catabolized denser HDL particles causing a reduction in serum HDL concentrations [25]. Alternatively, larger HDL particles exchange TG for the CE found in LDL particles by CETP [19]. The resultant TG-enriched LDL particles are delipidated by HL to generate sd-LDL particles that are potentially more atherogenic than larger LDL particles. VLDL metabolites generated through this pathway (sd-LDL particles in particular) remain in circulation for extended periods due to conformational changes of ApoB-100 that inhibit clearance [26].

In summary, synthesis and metabolism of small VLDL particles is associated with low serum TG levels, variable LDL-C levels, and higher serum HDL-C levels. In contrast, synthesis and metabolism of large VLDL particles is associated with higher serum TG levels, lower HDL-C levels, and generation of sd-LDL often within the context of "normal" serum LDL-C levels, a lipid triad termed "atherogenic dyslipidemia" [14, 21, 24].

3.4. Lipoproteins and Atherosclerosis

Lipoprotein particles are integral to initiation and progression of CHD, a disease characterized by lipid accumulation within large and medium-sized arteries with accompanying inflammation and fibrosis [27, 28]. Endothelial

cell (EC) injury and disruption caused by hypertension, diabetes, smoking, and dyslipidemia initiates CHD. Lipoproteins, LDL particles in particular, diffuse through EC junctions and are retained within the intima through proteoglycan/ApoB-100 interactions where constituent Apos and lipids undergo enzymatic and nonenzymatic oxidation [29]. Oxidized LDL particles release PL, activating EC inflammatory responses. These responses include the expression of vascular cell adhesion molecule-1 that binds monocytes as they roll along the vascular surface, and monocyte chemotractant protein-1 that stimulates monocyte migration into the intima [30]. A critical step in CHD occurs within the intima where monocyte-colony-stimulating factor, secreted by EC and vascular smooth muscle cells, transforms monocytes into resident macrophages [27, 28].

Intimal macrophages further contribute to LDL oxidation through enzymatic production of reactive oxygen species that oxidize LDL particle components [31]. Oxidized LDL particles are strong ligands for scavenger receptors expressed on macrophages, and as cholesterol and lipids contained within LDL particles amass in cytosolic droplets, macrophages are transformed into lipid-rich foam cells that accumulate to produce visible yellow fatty streaks within arterial walls [32]. Apoptotic foam cells release lipids that pool within the subintimal space at the center of an atherosclerotic plaque; the evolving plaque may protrude into the arterial wall, reducing blood flow to tissues supplied by the affected vessel [27, 28]. For example, in the coronary arteries, plaque formation leads to angina pectoris; in the carotid artery, transient ischemic attacks; and in the femoral artery, intermittent claudication. Plaque rupture or fissuring, caused in part by inflammatory reactions initiated by oxidized lipoprotein components of the plaque, results in thrombus formation and "acute" clinical events, including myocardial infarction, unstable angina, stroke, and death [33]. LDL particles are the most abundant atherogenic lipoproteins found in serum. However, it remains uncertain which lipoprotein markers best correlate with CHD-related outcomes.

4. LDL Particles

4.1. LDL Cholesterol

4.1.1. *LDL Cholesterol and CHD Risk*

It is well-established that elevated serum LDL-C concentrations are an independent predictor of CHD risk [34, 35]. Accordingly, ATP-III screening and therapeutic guidelines emphasize LDL-C as the most clinically useful LDL-related CHD predictor [2]. The current threshold LDL-C levels, derived from the Framingham Heart Study data and extended to the global

population, are relatively arbitrary: CHD disease risk varies substantially between individuals with similar LDL-C concentrations, and many individuals with "target" LDL-C levels suffer CHD-related events [36]. Indeed, a global LDL-C threshold for CHD risk has not been identified. Consequently, the current threshold levels often fail to identify individuals at increased CHD risk, particularly in groups underrepresented in the Framingham Offspring Study (younger individuals, women, and ethnic minorities) [36]. NCEP considers serum LDL-C to be a marker of circulating LDL particles, yet the accuracy of this correlate is dependent upon homogenous cholesterol content of LDL particles across populations. The presence of larger, cholesterol-enriched particles will result in an overestimation of particle numbers, whereas the presence of small, cholesterol-depleted particles will result in underestimation of particle numbers. Owing to the heterogeneity of LDL particles, individuals with similar serum LDL-C values may have particles of dissimilar size and number, factors that may explain the relative imprecision of LDL-C in predicting CHD risk in an individual patient. The variance between serum LDL-C concentrations and LDL particle numbers is illustrated by the Framingham Offspring Study in which LDL particle numbers were elevated above LDL-C in the context of "suboptimal" HDL-C concentrations (Fig. 2), underscoring that LDL-C underestimates CHD risk in subgroups of the population [37]. CHD risk classification is further confounded by the approximation method commonly used to estimate serum LDL-C levels.

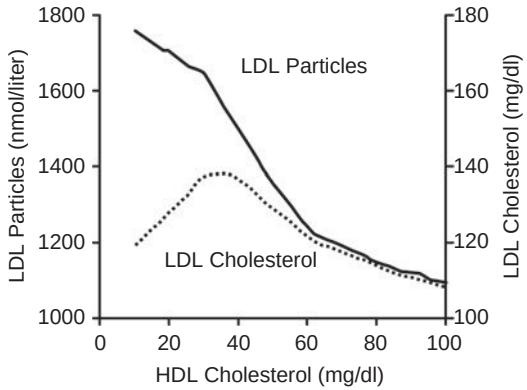

FIG. 2. Relationships between LDL cholesterol and LDL particle number in the Framingham Offspring Cohort.

4.1.2. *LDL Cholesterol Approximation*

Despite the importance attributed to LDL-C values in CHD risk assessment, LDL-C is approximated, rather than measured directly, using the Friedewald equation developed in the 1970s [38]:

$$\text{LDL-C} = (\text{TC}) - (\text{HDL-C}) - (\text{TG}/5)$$

Serum TC, HDL-C, and TG are measured using standardized enzyme-based methods [39, 40], and the influence of VLDL-associated cholesterol is adjusted with a correction term of TG/5 [38]. The accuracy of LDL-C approximation is dependent upon several critical assumptions, including serum TG levels <400 mg/dl and a consistent TG/VLDL-C ratio of "5," despite interindividual heterogeneity in VLDL particle lipid content [38]. Although the Friedewald equation is meant to estimate serum LDL-C, it actually reflects the cholesterol content of all ApoB lipoproteins, including cholesterol found in VLDL, VLDL remnants, IDL, LDL, and Lp(a) [41].

Estimated LDL-C values may be inaccurate in individuals with low serum HDL-C [42]. In addition, LDL-C is also underestimated in individuals with metabolic syndrome, a condition that affects approximately 24% of the population and is characterized by elevated TG concentrations [43]. Given the prevalence of metabolic syndrome, underestimation of LDL-C levels may be widespread, leading to misclassification of CHD risk. Inaccurate LDL-C estimates have also been observed in diabetic populations: only 49% [44] to 68% [45] of patients with diabetes had a Friedewald LDL-C value within 10% of directly measured LDL-C. Friedewald estimations of LDL-C are also unreliable in individuals with liver disease [46], nephrotic syndrome [47] and chronic renal insufficiency [48, 49], and in women taking hormone replacement therapy [50].

On the basis of the limitations associated with LDL-C estimation, the NCEP recommends phasing out the use of Friedewald equation in favor of direct LDL-C measurement. The high prevalence of metabolic syndrome and the possible inaccuracy of estimated LDL-C in this setting led the ATP-III to recommend calculation of non-HDL-C as a secondary lipid target in individuals with serum TG between 200 and 499 mg/dl [2]:

$$\text{non-HDL-C} = (\text{TC-HDL-C})$$

Non-HDL-C is a simple measure of the cholesterol load associated with all atherogenic ApoB lipoproteins. At present, despite potential inaccuracies, estimated LDL-C is routinely used for risk assessment given the availability of population-based reference ranges. However, the limitations associated with LDL-C estimation have stimulated the search for alternative measures of LDL particle atherogenicity that may provide incremental information about CHD risk, including LDL particle size and number.

4.2. LDL Particle Size

LDL peak particle size exhibits a bimodal distribution and can be divided into distinct phenotypes that differ in physical properties, metabolism, and, potentially, atherogenicity [14]. Phenotypes are classified as Pattern A (larger, buoyant LDL particles), Pattern B (smaller, denser LDL particles), or intermediate (both patterns A and B) [51, 52]. Genetic factors account for a large proportion of interindividual variation in LDL particle size [53], and linkage studies have identified genomic sequences that influence LDL particle size [54]. In addition, sd-LDL prevalence increases with age: Pattern B is prevalent in approximately 5–10% of men and women less than 20 years of age, 30% of adult men, and 15–25% of postmenopausal women [51, 55]. LDL particle size correlates positively with serum HDL-C levels and inversely with serum TG levels [56]. A defining characteristic of the metabolic syndrome is atherogenic lipoprotein phenotype [51, 52], dyslipidemia characterized by sd-LDL particles, reduced HDL levels, and elevated TG levels.

The ATP-III recognizes LDL particle size as an emerging CHD risk factor [2]. The increased atherogenicity of sd-LDL particles is supported by the *in vitro* and *in vivo* observations that sd-LDL undergoes greater transendothelial transport [57, 58], greater interaction with matrix proteoglycans [59], and greater susceptibility to oxidation [60, 61] as compared to larger LDL particles. In addition, the majority of cross-sectional and prospective epidemiological studies have shown significant associations between sd-LDL and CHD risk [4, 62]. Other than in the Quebec Cardiovascular Study [63], sd-LDL particle size has not been found to be an independent predictor of risk after multivariate adjustment for serum TG and HDL-C levels [4]. However, lack of statistical significance in multivariate models that include TG and HDL-C (and thus may be affected by colinearity) does not exclude a causal role of small LDL particle size in CHD development. LDL particle size measurement may be useful in assessing risk in select patients, choosing appropriate lipid-lowering agents, and monitoring efficacy of diet, exercise, and lipid-lowering therapy. LDL particle size is assessed following physical subclass separation by particle density, size, or chemical composition utilizing ultracentrifugation, electrophoresis, and precipitation, while nuclear magnetic resonance (NMR) spectroscopy measures particles size independent of physical properties.

4.2.1. *Ultracentrifugation*

4.2.1.1. *Sequential Ultracentrifugation.* Sequential ultracentrifugation, first described by Havel *et al.* [64] in the 1950s, was the first technique used to isolate the major lipoprotein classes and remains the gold standard for measuring lipoprotein particle size. Ultracentrifugation effectively resolves

lipoproteins from other serum proteins based on differences in hydrated particle densities, and further separation of each lipoprotein class is possible due to differences in lipid and protein content [65]. In a sequential ultracentrifugation experiment, the biological sample density is determined and adjusted to equal the density of the lipoprotein class of interest. The sample undergoes 18–24 hours of ultracentrifugation between 40,000 and 100,000*g*, forcing particles denser than the solvent to sink to the bottom of the sample whereas less dense particles rise to the surface and are collected by capillary pipette. This procedure is repeated until each lipoprotein class has been isolated. Following ultracentrifugation, the cholesterol and Apo content of each lipoprotein fraction can be analyzed.

Ultracentrifugation offers limited clinical utility because it is time consuming and labor intensive. Moreover, subsequent analysis of lipoprotein fractions may be compromised as Apos tend to redistribute among lipoprotein particles or become oxidized during sample processing [65].

4.2.1.2. *Single Vertical Spin Autoprofiling.* More recently, a variant of density ultracentrifugation, vertical spin autoprofiling (VAP), has been developed to profile lipoprotein classes [66]. VAP separates lipoprotein classes with a short single vertical spin ultracentrifugation, followed by continuous-flow enzymatic analysis of cholesterol and TG by an autoanalyzer [67]. Absorbance data are continually analyzed throughout data collection, and cholesterol values from each lipoprotein class are integrated to obtain a spectrophotometric tracing of each lipoprotein subclass pattern. Thus, a cholesterol profile is constructed and decomposed into curves corresponding to lipoprotein classes. VAP measures cholesterol in 18 lipoprotein subclasses that include large, buoyant LDL (LDL-2 and LDL-1), intermediate LDL (LDL-4 and LDL-3), and sd-LDL (LDL-5) [68].

VAP has fair precision and reproducibility, with an intraassay coefficient of variation less than 10% [68]. In addition, data derived using VAP correlate strongly with gradient gel electrophoresis (GGE; $r = 0.86$) [68].VAP has several advantages over traditional ultracentrifugation techniques, including relatively high-throughput analysis and reduced artifact formation. In addition to LDL particle size data, VAP provides serum TC and HDL-C concentrations similar to conventional enzymatic assays as well as a direct LDL-C measurement, superior to the estimations provided by Friedewald equation. VAP technology can be performed through a contracted service or within an on-site laboratory.

4.2.2. *Electrophoresis*

GGE was developed in the 1970s in response to the need for a simple yet accurate method to measure lipoprotein particle size [69]. Differences in size and surface charge among lipoprotein classes permit adequate separation by

electrophoresis to yield distinct regions in which VLDL, IDL, LDL, HDL, and their constituent subclasses reside [13, 70–72]. Notably, GGE can distinguish between large-LDL, intermediate-LDL, and sd-LDL particles.

4.2.2.1. *Gradient Gel Electrophoresis.* Serum samples and assay standards are loaded onto gradient polyacrylamide gels (3–16%) and electrophoresed for over 24 hours. Proteins on the gels are stained with Coomassie blue protein dye and then destained with a methanol/acetic acid solution. For lipid staining, gels are stained with Sudan Black B overnight and then destained with acetic acid. The gels are then scanned, migration distances from the sample well to assay standards are measured, and relative migration distance is calculated. Particle size is determined by plotting relative migration of standards against their known hydrated densities. The resulting regression equation is used to estimate lipoprotein particle size from the relative migration of each lipoprotein particle peak and is classified as small (diameter $<$25.8 nm), intermediate (diameter 25.8–26.3 nm), or large (diameter $<$26.3 nm) [13, 70–72].

The requisite equipment for GGE is relatively inexpensive, the gels are commercially available, and lipoprotein resolution is excellent. However, the process and analysis associated with GGE are time and labor intensive, and thus sample throughput is low, rendering GGE impractical for clinical applications.

4.2.2.2. *Tube Gel Electrophoresis.* Polyacrylamide tube gel electrophoresis is an improved electrophoretic method that measures LDL subclass size in less than 3 hours [73]. The system uses high-resolution precast 3% polyacrylamide gel tubes for electrophoresis. The biological sample is mixed with a liquid loading gel that contains Sudan Black B to stain lipoprotein lipid components. This mixture is then added to the polyacrylamide gel tubes and allowed to polymerize for 30 min in front of a fluorescent light source. Samples are then electrophoresed for 1 hour at 3 mA per tube, and densitometry is performed at 610 nm. In this method, LDL is subfractionated into seven bands with 1–2 designated as large LDL and 3–7 as sd-LDL (Fig. 3).

Two different quantification methods are used in conjunction with the tube gel electrophoresis method. Using an automated, computer-assisted scoring system [73], the densitometry data are combined with the electrophoretic scan pattern of size-fractionated LDL particles to calculate a subfractionation score and assign a phenotype. The scoring system is based on average particle size distribution to describe the profile: a score $>$8.5 indicates sd-LDL, 5.5–8.5 intermediate LDL, and $<$5.5 large LDL. This analysis method has a good general agreement with particle assignments generated by GGE and NMR spectroscopy [73]. Alternatively, following electrophoresis and densitometry, the slowest migrating VLDL band is assigned an Rf value of 0,

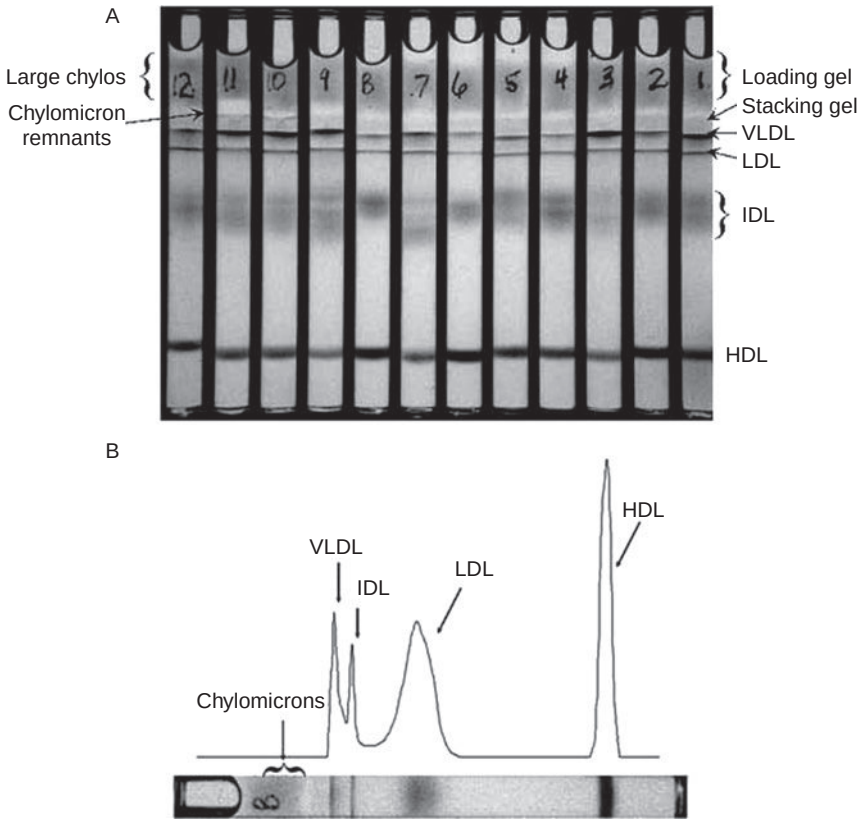

FIG. 3. Tube gel electrophoresis. (A) Separation of serum lipoproteins using tube gel electrophoresis. (B) Lipoprotein particle distribution using the analysis method of Hoefner *et al.* [75].

and the fastest migrating HDL band is assigned an Rf value of 1 [74]. The Rf values of the LDL subfraction bands are then calculated as follows:

$$\frac{\text{distance between VLDL and LDL subfraction bands}}{\text{distance between VLDL and HDL bands}}$$

The LDL subclasses are assigned as sd-LDL ($Rf > 0.4$), intermediate LDL (Rf 0.38–0.40), or large LDL ($Rf < 0.38$). This analysis method correlates reasonably with GGE ($r = 0.78$).

The major advantages of tube gel electrophoresis are high throughput, technical simplicity, and precision. A limitation is that each analysis system described has different cut-points for LDL particle sizes, and thus, values cannot be used interchangeably. This method may be suitable for routine

clinical quantification of LDL particle size, but standardization of analysis methods will be required.

4.2.3. *Precipitation*

Lipoprotein separation through precipitation techniques was first introduced in the 1970s [38, 75]. Today, revised protocols allow isolation of lipoprotein fractions within hours. Precipitation methods have conventionally been used to isolate HDL lipoproteins prior to measurement of cholesterol [76], and, more recently, precipitation has also been applied to measurement of LDL particle size.

4.2.3.1. *Denka Method.* The Denka method measures sd-LDL utilizing precipitation techniques followed by immunoturbidometric quantification of ApoB-100 levels [77]. ApoA lipoproteins and higher-density ApoB particles ($D > 1.044$ g/ml) are precipitated from a biological sample through addition of a heparin-sodium salt and magnesium chloride precipitation reagent. Following a brief centrifugation, the sample yields a tightly packed precipitate and a supernatant that contains sd-LDL. The supernatant is sampled, and ApoB is measured by immunoturbidometry to provide direct quantification of sd-LDL particle number. Cholesterol associated with sd-LDL can also be measured with the homogenous method. This technique allows a direct, high-throughput measurement of serum sd-LDL levels. The Denka method correlates strongly with ultracentrifugation values for measurement of both sd-LDL-C levels ($r = 0.884$) and LDL ApoB level ($r = 0.89$) [78].

4.2.4. *NMR Spectroscopy*

NMR spectroscopy is able to distinguish between at least 15 lipoprotein subclasses, including large LDL (L3), intermediate LDL (L2), and sd-LDL (L1), while simultaneously measuring both lipoprotein particle size and quantity [79]. NMR spectroscopy depends upon spectroscopic distinctness across lipoprotein subclasses; each lipoprotein subclass displays a unique peak at a characteristic frequency that is dependent upon particle size. The protons on terminal methyl groups of lipids emit signals that integrate to form a bulk signal for each lipoprotein particle present, and the amplitude of these peaks indicates the quantity of particles present in the sample. In addition to lipoprotein particle numbers, NMR spectroscopy provides an accurate measure of serum cholesterol and TG concentrations. Importantly, a strong correlation ($r = 0.91$) has been found between LDL-C measured by NMR spectroscopy and enzymatic methods. NMR spectroscopy directly measures lipoprotein and lipid content, and it is also clinically feasible due to its automated, rapid processing of nonfasting samples. Although NMR spectroscopy is an inexpensive measure to accurately determine multiple

lipoprotein characteristics, the initial equipment investment is high and may be beyond the resources for many clinical laboratories.

Recently, Ensign *et al.* [80] compared diagnostic LDL phenotype classifications (Pattern A, large LDL particles; Pattern AB, intermediate LDL particles; or Pattern B, sd-LDL particles) assigned by clinical labs following VAP, GGE, tube gel electrophoresis, and NMR spectroscopy. The results were highly disparate between the four methods as phenotype classification was identical in only 8% of the samples. Notably, the highest interlaboratory agreement was between GGE and NMR methodologies (>70%). These results underscore the need to standardize methods that assess LDL size. However, the ability to standardize such diverse assays is questionable as lipoprotein subclasses and sizes differ between methods. At present, the clinical utility of LDL subclass determination is hampered by the lack of standardization among different assays.

4.3. LDL Particle Number

4.3.1. *LDL Particle Number and CHD Risk*

LDL particle numbers may be of greater importance in CHD risk prediction than are either serum LDL-C levels or LDL particle size as atherogenic particle number is the most important determinant of the likelihood that lipoproteins will traverse the arterial endothelium and contribute to atherogenesis [81]. Notably, particle numbers predict CHD risk independently of other lipid risk factors to a greater extent than LDL-C levels in a series of prospective epidemiological studies [63, 82–93]. For instance, the Apolipoprotein-related Mortality Risk Study demonstrated that ApoB concentrations had higher sensitivity and specificity in predicting fatal myocardial events as compared to LDL-C [85, 93]. Furthermore, study of the Insulin Resistance Atherosclerosis Study population found that the magnitude of correlation between ApoB and CHD risk factors was greater than that between LDL-C, even after adjustment for LDL-C and the Framingham risk score, whereas the magnitude of association between LDL-C and CHD risk factors was weakened by comparable adjustment [94]. In contrast, the Atherosclerosis Risk in Communities Study did not show an independent association of ApoB-100 levels with CHD risk after adjustment for traditional lipid risk factors [95], a finding that may have resulted from the use of a nonstandardized ApoB assay with a relatively large coefficient of variation.

Measurement of LDL particle numbers has been proposed as a candidate CHD risk factor [3], and LDL particle numbers can either be approximated through the calculation of non-HDL-C or measurement of structural Apos, or measured directly using NMR spectroscopy.

4.3.2. *Non-HDL Cholesterol*

Calculation of non-HDL-C has been proposed by the ATP-III as an estimate of the atherogenic burden associated with lipoprotein particle numbers as it has been found to correlate with ApoB measurements in more than a dozen studies, and calculation does not require an additional expense [2]. However, the strength of this correlation diminishes as TG concentrations rise [96] and at HDL-C concentrations <40 mg/dl [37], thereby reducing the predictive efficacy of non-HDL-C in a substantial proportion of the population.

4.3.3. *ApoB Measurement*

Measurement of serum ApoB-100 provides an estimate of LDL particle number since ApoB-100 lipoprotein particles each contain one ApoB-100 molecule, and approximately 90% of ApoB-100 particles recovered in serum are associated with LDL [97, 98]. ApoB-100 can be measured using various immunoassay techniques, including radioimmunoassay, enzyme-linked immunoassays, and immunoturbidometric assays, the latter being frequently employed in clinical laboratories. In these assays, serum is combined with specific antihuman Apo antibodies, and the binding of antibodies to serum Apos forms an insoluble aggregate in solution that manifests as turbidity. The degree of turbidity in the sample is proportional to apolipoprotein concentration and is measured spectrophotometrically using an autoanalyzer. In addition to reflecting concentrations of LDL particles, calculation of the LDL-C: ApoB-100 ratio may provide an estimate of LDL particle size; a ratio of less than 1.2 indicates a preponderance of sd-LDL in serum [77]. The key advantages of immunoturbidometric assays are technical simplicity, robust results, and high precision [99]. Further advantages include commercial availability of test reagents, the widespread use of autoanalyzers in clinical settings, and the availability of standardized reference materials [100, 101]. ApoB-100 measurement provides information about LDL-associated atherogenicity beyond estimation of LDL-C alone. However, results must be interpreted in the context of plasma TG levels and standardized population-based reference values that would allow consistent data interpretation are not yet available.

The ATP-III guidelines advocated use of non-HDL-C over ApoB measurement. However, at the time of publication of these guidelines, a standardized clinical assay for ApoB measurement was not widely available, measurement of ApoB would have required an additional cost, and the superior predictive power of ApoB to non-HDL-C had not yet been sufficiently demonstrated [2]. The Insulin Resistance Atherosclerosis Study subsequently showed that after adjustment for non-HDL-C levels, ApoB retained a strong correlation with conventional CHD risk markers, whereas

adjustment of non-HDL-C for ApoB levels eliminated a correlation between non-HDL-C and CHD risk factors [102]. Thus, the utility of non-HDL-C as a surrogate marker for atherogenic particle number is debatable. ApoB-100 concentrations provide a reasonable estimation of atherogenic LDL particle number, and based on the substantial evidence that ApoB-100 levels provide predictive value of CHD risk beyond LDL-C, the Canadian Cardiovascular Society [103] and the Canadian Diabetes Association [104] have introduced ApoB as an alternative to LDL-C measurement in their most current cardiovascular disease prevention guidelines. Furthermore, standardized ApoB-100 assays and reference materials are now available [100, 101]. However, studies that have evaluated both ApoB-100 approximated and NMR spectroscopy-measured LDL particle numbers have shown the latter to be a superior predictor of CHD-related events [83, 92]. Presently the start-up costs of NMR are cost prohibitive for many laboratories, and common reference materials are not yet available. Although LDL particle number is an important measure of atherogenicity, the ideal measurement methodology is yet to be established [3, 62].

4.4. Lipoprotein (a)

4.4.1. *Lipoprotein(a) and CHD Risk*

Lp(a), an LDL variant, has been proposed as a CHD risk marker [2], and the atherogenicity associated with Lp(a) particles has been attributed to the molecule's enhanced capacity to traverse the arterial endothelium, the large cholesterol pool, and the reduced affinity for LDL-R-mediated clearance from circulation [105]. A meta-analysis of 18 prospective studies found that subjects in the highest tertile of Lp(a) levels had significantly increased CHD risk as compared to subjects in the lowest tertile [106], a finding that was confirmed in a later prospective study of more than 9,000 subjects [107].

4.5. Lp(a) Measurement

Lp(a) is also measured using immunoturbidometric assays, in which Apo(a) is detected using polyclonal antibodies directed against repeated motifs (Kringle IV domains), within the Apo(a) moiety. Ten different Kringle IV sequences (patterns 1–10) are recognized by antibodies, with patterns 1 and 3–10 present in a single copy and pattern 2 present in variable copy numbers from 3 to >40 [10]. The structural complexity of Lp(a) molecules renders accurate measurements difficult as values obtained using polyclonal antibodies are skewed by the number of Kringle IV Pattern 2 repeats; smaller Apo(a) isoforms with fewer repeats may be underestimated while larger isoforms

with increased Pattern 2 copy numbers may be overestimated [108]. Development and standardization of high-throughput methods utilizing antibodies insensitive to Lp(a) Kringle IV Pattern 2 repeats are required for precise lipoprotein measurement. Although immunoassays utilizing monoclonal antibodies are an alternative, a limitation is that oxidative modification of the Apo(a) moiety may reduce antibody binding, leading to underestimation of serum Lp(a) concentrations [109]. An additional challenge to accurate quantification of serum Lp(a) values is the lack of standardized reference materials available for assay calibration. Calibrators-assigned Lp(a) values based on the mass ratio of Apo(a)/ApoB yield variable results due to Lp(a) size heterogeneity, signifying that Lp(a) values associated with assay calibrators, and consequently, serum samples should be expressed in terms of molarity [109].

Serum Lp(a) levels are considered an independent CHD risk marker by the ATP-III, but due to methodological limitations and expense, Lp(a) screening is currently recommended only for patients with early onset CHD or family history of CHD [2]. Lp(a) values should be reported as a percentile of the general population, and increased risk assigned to patients with Lp(a) levels greater than the 80th percentile [109].

5. Conclusion

It is well established that LDL cholesterol is important in the development of CHD. However, measurement of cholesterol associated with LDL particles may underestimate risk in individuals with small LDL particles and in those with an increased number of LDL particles. Measurement of LDL particle size and number may provide a better estimate of CHD risk than LDL cholesterol alone. Routine measurement of LDL particle size is not recommended in part because methods to measure LDL particle size, including ultracentrifugation, GGE, capillary gel electrophoresis, and NMR spectroscopy, have yet to be standardized, common reference materials are unavailable, and disparity is observed between the various methods. With regard to LDL particle number, ApoB quantification using standardized immunoturbidometric assays may provide incremental information about CHD risk as well as efficacy of lipid-lowering drugs and has been included in the Canadian national guidelines for CHD prevention. Measurement of Lp(a) is not recommended in the general population because of methodological limitations, including antibody specificity and lack of common reference materials. In spite of these limitations, measurement of Lp(a) may be clinically useful in patients with early onset CHD or family history of CHD. Further work is needed to refine assays that measure LDL particle modifications (oxidation

of protein and lipid components) and assess their utility in CHD risk assessment. An area of active investigation is whether HDL particle size, functionality, and chemical modification provide clinically relevant information beyond HDL-C levels. Further prospective studies will be needed to identify which lipoprotein parameters and measurement methodologies best predict CHD risk.

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