Academic Press is an imprint of Elsevier

525 B Street, Suite 1800, San Diego, CA 92101-4495, USA
225 Wyman Street, Waltham, MA 02451, USA
32 Jamestown Road, London NW1 7BY, UK
The Boulevard, Langford Lane, Kidlington, Oxford, OX5 1GB, UK
Radarweg 29, PO Box 211, 1000 AE Amsterdam, The Netherlands

First edition 2014

ISBN: 978-0-12-800141-7
ISSN: 0065-2423

Printed and bound in United States of America
14 15 11 10 9 8 7 6 5 4 3 2 1

CONTRIBUTORS

Francisco V. Álvarez Menéndez
Clinical Biochemistry, Laboratory of Medicine, Hospital Universitario Central de Asturias, and Biochemistry and Molecular Biology Department, University of Oviedo, Oviedo, Spain

Kaj Blennow
Clinical Neurochemistry Laboratory, Institute of Neuroscience and Physiology, The Sahlgrenska Academy at the University of Gothenburg, Mölndal, Sweden

Fatima R. Freitas
Lipid Metabolism Laboratory, Heart Institute (InCor), Medical School Hospital, University of São Paulo, São Paulo, Brazil

Gregory T. Jones
Department of Surgery, Dunedin School of Medicine, University of Otago, Dunedin, New Zealand

Romy Kursawe
Department of Pediatrics, Yale University School of Medicine, New Haven, Connecticut, USA

Ronald Lautner
Clinical Neurochemistry Laboratory, Institute of Neuroscience and Physiology, The Sahlgrenska Academy at the University of Gothenburg, Mölndal, Sweden

Rui-Chao Lin
School of Chinese Materia Medica, Beijing University of Chinese Medicine, Beijing, PR China

Robert J. Lock
Immunology and Immunogenetics, Pathology Sciences, Southmead Hospital, North Bristol NHS Trust, Bristol, United Kingdom

Raul C. Maranhão
Lipid Metabolism Laboratory, Heart Institute (InCor), Medical School Hospital, and Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil

Niklas Mattsson
Clinical Neurochemistry Laboratory, Institute of Neuroscience and Physiology, The Sahlgrenska Academy at the University of Gothenburg, Mölndal, Sweden; Department of Veterans Affairs Medical Center, Center for Imaging of Neurodegenerative Diseases, and Department of Radiology and Biomedical Imaging, University of California, San Francisco, California, USA

Belén Prieto
Clinical Biochemistry, Laboratory of Medicine, Hospital Universitario Central de Asturias, Oviedo, Spain

Christoffer Rosén
Clinical Neurochemistry Laboratory, Institute of Neuroscience and Physiology, The Sahlgrenska Academy at the University of Gothenburg, Mölndal, Sweden

Nicola Santoro
Department of Pediatrics, Yale University School of Medicine, New Haven, Connecticut, USA

Pashtun Shahim
Clinical Neurochemistry Laboratory, Institute of Neuroscience and Physiology, The Sahlgrenska Academy at the University of Gothenburg, Mölndal, Sweden

Tobias Skillbäck
Clinical Neurochemistry Laboratory, Institute of Neuroscience and Physiology, The Sahlgrenska Academy at the University of Gothenburg, Mölndal, Sweden

David J. Unsworth
Immunology and Immunogenetics, Pathology Sciences, Southmead Hospital, North Bristol NHS Trust, Bristol, United Kingdom

Henrik Zetterberg
Clinical Neurochemistry Laboratory, Institute of Neuroscience and Physiology, The Sahlgrenska Academy at the University of Gothenburg, Mölndal, Sweden, and UCL Institute of Neurology, Queen Square, London, United Kingdom

Ying-Yong Zhao
Key Laboratory of Resource Biology and Biotechnology in Western China, Ministry of Education, The College of Life Sciences, Northwest University, Xi'an, Shaanxi, PR China

PREFACE

The third volume of the Advances in Clinical Chemistry series for 2014 is presented.

In Chapter 1, the intravascular metabolism of HDL cholesterol is examined. The role of lipid transfer via cholesteryl ester and phospholipid transfer proteins is examined with respect to risk of cardiovascular disease as well as the atheroprotective properties of HDL cholesterol. In Chapter 2, diagnosis of sepsis, a major cause of death in the acute care setting is reviewed. Differentiation of infectious process in systemic inflammatory responses is crucial to rapid identification and treatment. In Chapter 3, the complex molecular and pathophysiologic mechanisms leading to nephrotoxicity are explored. The role of metabolomics for screening a wide array of kidney injury biomarkers is presented. In Chapter 4, metabolic syndrome in pediatric patients is examined. The unique aspects of this syndrome in childhood obesity are presented and the role of novel molecules and biomarkers in its pathogenesis are explored. In Chapter 5, the neuropathology of Alzheimer's disease is reviewed. The development and use of classical and novel cerebrospinal fluid biomarkers are presented. Standardization of these analytical approaches is critical for highly specific and sensitive diagnostics. In Chapter 6, food allergy IgE mediated hypersensitivity, a growing problem in the Western world, is examined. Appropriate screening tools and standardized methods are crucial to prevent this potentially life threatening condition and aid treatment. In Chapter 7, the importance of biologic sampling in matrix metalloproteinases is presented. These endopeptidases appear to have an increasing role in many physiologic and pathophysiologic processes. A comprehensive understanding of endogenous metabolism, activation of proforms and preanalytical considerations is vital in understanding their role in disease states.

I thank each contributor of Volume 65 and extend thanks to colleagues who contributed to the peer review process. I would also like to thank Helene Kabes for her expert editorial support at Elsevier.

I hope the third volume for 2014 will be enjoyed. Your comments and feedback are always appreciated.

I would like to dedicate Volume 65 to my daughter Stephanie on the occasion of her 13th birthday.

GREGORY S. MAKOWSKI

CHAPTER ONE

HDL Metabolism and Atheroprotection: Predictive Value of Lipid Transfers

Raul C. Maranhão[*,†,1], **Fatima R. Freitas**[*]
[*]Lipid Metabolism Laboratory, Heart Institute (InCor), Medical School Hospital, University of São Paulo, São Paulo, Brazil
[†]Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil
[1]Corresponding author: e-mail address: ramarans@usp.br

Contents

Abstract

High-density lipoprotein (HDL) intravascular metabolism is complex, and the major HDL functions are esterification of cholesterol and reverse cholesterol transport, in which cholesterol from cells is excreted in bile. HDL has also several other antiatherogenic functions: antioxidative, vasodilatatory, anti-inflammatory, antiapoptotic, antithrombotic, and anti-infectious. Low HDL cholesterol is a major risk factor for cardiovascular disease (CVD) and high HDL cholesterol may favor the many protective abilities of HDL. However, aspects of HDL function can be independent of HDL cholesterol levels, including the efflux of cholesterol from cells to HDL. Some populations show low incidence of CVD despite their low HDL cholesterol. Lipid exchange between HDL and other lipoproteins and cells is fundamental in HDL metabolism and reverse cholesterol transport. By determining HDL composition, lipid transfers can also affect HDL functions that depend on proteins that anchor on HDL particle surface. Cholesteryl ester protein

Advances in Clinical Chemistry, Volume 65
ISSN 0065-2423
http://dx.doi.org/10.1016/B978-0-12-800141-7.00001-2

(CETP) and phospholipid transfer protein facilitate lipid transfers among lipoprotein classes, but the role of the lipid transfers and transfer proteins in atherosclerosis and other diseases is not well established. CETP has become a therapeutic target because CETP inhibitors increase HDL cholesterol, but to date the clinical trials failed to show benefits for the patients. Recently, we introduced a practical *in vitro* assay to evaluate the simultaneous transfer from a donor nanoemulsion to HDL of unesterified and esterified cholesterol, phospholipids, and triglycerides. Groups of subjects at different clinical, nutritional, and training conditions were tested, and among other findings, lower transfer ratios of unesterified cholesterol to HDL were predictors of the presence of CVD.

1. INTRODUCTION

An important aspect of lipoprotein metabolism is that the lipoprotein particles continuously exchange their constituent lipids and proteins. Lipid transfers among lipoprotein particles may enrich or deplete lipoprotein classes of given lipid species. Lipid transfers are determinant of changes in the composition and metabolism of lipoproteins. The implications of those process in the genesis of atherosclerosis and other degenerative diseases are still largely unknown and lead to a fascinating avenue of research.

The initial observations on the exchange of phospholipids and cholesterol between lipoproteins were made by Hagerman *et al.* during investigations on lipid exchanges between membranes published in 1951 [1]. The shift of cholesteryl esters from high-density lipoprotein (HDL) to low-density lipoprotein (LDL) was firstly documented by Rehnborg and Nichols in 1964 [2]. Zilversmit *et al.* reported the protein-mediated nature of the lipid transfers [3]. Those authors showed that an acidic protein stimulated the transfer of cholesteryl esters between VLDL and LDL. This protein was described as a large molecular weight glycoprotein, which promotes the exchange of cholesteryl esters between all classes of lipoproteins in the human plasma [4]. In 1980, Ihm *et al.* found another glycoprotein that also mediates the exchange of phospholipids between lipoproteins [5]. At present, it is established that the main proteins involved in lipid transfers are cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP). CETP facilitates the transfer of cholesteryl esters, triglycerides, and phospholipids, whereas PLTP mostly favors the transfers of phospholipids [6]. Although the transfer of unesterified cholesterol is less influenced by transfer proteins, it may also be increased by PLTP action [7].

CETP mediates the transfer of triglycerides from the triglyceride-rich lipoproteins such as VLDL to HDL and, in the opposite direction, the

transfer of cholesteryl ester from the core of HDL particles to VLDL [6]. In fact, those lipid shifts are bidirectional but the result is depletion of cholesteryl esters in HDL and enrichment of HDL with triglycerides. This leads to the formation of large HDL particles that are an appropriate substrate for hepatic lipase and removal by the liver [8]. In hypertriglyceridemia, accumulation of VLDL and other triglyceride-rich lipoproteins in the blood leads, by mass action law, to increase in HDL triglyceride content and increased HDL clearance by the liver consequent to the hepatic lipase action. Resulting from this chain of events is the so-called triglyceride–HDL cholesterol seesaw, a phenomenon that is commonly observed in the clinical practice: when serum triglycerides increase, HDL cholesterol decreases and vice versa [8]. It is worthwhile that the driving forces for the seesaw are the variations in VLDL and not in HDL concentration.

Although the process of lipid transfers involves all the lipoprotein classes, the effects on composition and metabolism are less important in regard to VLDL or LDL than to HDL, as exemplified above by the seesaw effect. The pharmacological inhibition of CETP results primarily in rise in HDL cholesterol concentration; LDL cholesterol decreases, but at much smaller rates. Rise of HDL cholesterol by CETP inhibition has become a new therapeutic target for antiatherosclerosis treatment. The fact that lipid transfers play a key role in HDL composition, concentration, and metabolism in the plasma suggests opportunities to establish new markers for HDL function and participation in pathological processes. A better comprehension of the implications of lipid transfers involving HDL is also necessary for the correct evaluation of pharmacological interventions on HDL metabolism.

2. HDL COMPOSITION

The HDL plasma fraction is constituted of spherical or discoidal particles of high hydrated density, ranging from 1.063 to 1.21 g/mL, due to elevated protein content (>30% by weight) compared with other lipoproteins [9]. Discoidal HDLs are the smallest HDL particles, constituted of apolipoprotein (apo) A-I embedded in a monolayer of phospholipids and free cholesterol [10]. Spherical HDL is larger and contains a hydrophobic core formed by cholesteryl esters and small amounts of triglycerides surrounded by a monolayer of phospholipids and unesterified cholesterol [9]. Apolipoproteins and a variety of other proteins in smaller amounts are anchored to the lipoprotein surface.

Apo A-I (MW: 28 kDa) is the major structural apolipoprotein of HDL and makes up approximately 70% of total HDL protein, whereas the second major HDL apolipoprotein, apo A-II, accounts for roughly 20%. The remaining 10% of the protein moiety includes apo E, apo A-IV, apo A-V, apo J, apo C-I, apo C-II, and apo C-III [11].

HDL particles also carry enzymes involved in lipid metabolism, such as lecithin-cholesterol acyltransferase (LCAT), with antioxidative activity, such as platelet-activating factor-acetyl hydrolase (PAF-AH), paraoxonase 1 (PON1), and glutathione selenoperoxidase (GSPx), and other proteins and peptides, such as serum amyloid A (SAA), a major positive acute-phase reactant, α-1-antitrypsin, a potent inhibitor of serine proteinases, and amyloid-β [9].

3. HDL SUBCLASSES

Within the HDL density range, there are heterogeneous groups of particles differing not only in density and size but also in lipid and protein composition and electrophoretic mobility [12]. Based on the physical–chemical characteristics, those HDL subclasses can be resolved by different techniques. By sequential ultracentrifugation, HDL can be separated into two main subfractions: large, light, lipid-rich HDL2 (density 1.063–1.125 g/mL) and small, dense, protein-rich HDL3 (1.125–1.21 g/mL). HDL2 and HDL3 can be further resolved by gradient gel electrophoresis into five distinct subpopulations, according to their size: HDL3c (7.2–7.8 nm), HDL3b (7.8–8.2 nm), HDL3a (8.2–8.8 nm), HDL2a (8.8–9.7 nm), and HDL2b (9.7–12.9 nm) [13]. By two-dimensional electrophoresis, it is possible to separate HDL by charge and size in more than 10 subspecies. HDL can also be separated on the basis of apolipoprotein composition into subpopulations by immunoaffinity methods [13].

The HDL subspecies profile reflects the overall steps of the lipoprotein metabolism and can be modified by myriads of variables in the factors that intervene in this metabolism, such as the lipid transfers, enzymatic activities, the kinetics, and concentration of the several lipoprotein classes and subclasses in the circulation, among many others.

4. HDL METABOLISM AND REVERSE CHOLESTEROL TRANSPORT

HDL metabolism is more complex than that of the other lipoprotein classes because, different from the apo B-containing lipoprotein classes,

HDL is mostly assembled in the plasma by lipidation of apo A-I secreted by the liver and the intestine [14,15] (Fig. 1.1).

HDL formation begins with the lipidation of apo A-I [10]. It is postulated that the initial phospholipidation of apo A-I occurs in the endoplasmic reticulum [16], and did not depend on the ATP-binding cassette A-I receptor (ABCA1), that is expressed mainly in the liver, intestine, and macrophages [17]. Subsequently, ABCA1 promotes the transport of phospholipids and unesterified cholesterol to lipid-poor apo A-I in the plasma, forming beta HDL, the discoid nascent form of HDL [18]. Unesterified cholesterol and phospholipids can also be transferred to nascent HDL apo A-I during the lipolysis of triglyceride-rich lipoproteins, such as VLDL and chylomicrons, as excess surface lipids detach from the shrinking lipoproteins [19].

The next important step is the esterification of HDL cholesterol by LCAT using apo A-I as cofactor [19]. LCAT is synthesized by the liver and circulates bound to lipoproteins, mainly to HDL, or as free, unassociated molecule [20]. Cholesteryl esters are more hydrophobic than unesterified cholesterol, so that after esterification cholesterol is dislocated from the surface lipoprotein monolayer to the core of the particles, where it is sequestered from the aqueous medium that surrounds the lipoprotein particle [21]. This results in the formation of the mature spherical HDL2 and HDL3 particles that form the bulk of the HDL plasma fraction.

Cholesterol esterification prevents the spontaneous back exchange of cholesterol from HDL to cells and thus promotes the net cellular removal of cholesterol [21]. Cholesterol esterification creates a gradient for the lipoprotein surface to receive additional unesterified cholesterol from the other lipoproteins and cells. On the other hand, it stabilizes the cholesterol plasma pool by avoiding escape of unesterified cholesterol from the surface lipoprotein layer to the plasma, to where it could easily diffuse and precipitate. In fact, although cholesterol esterification also occurs at some degree in other lipoprotein fractions, the HDL fraction is the site wherein most of the process takes place. This occurs because the HDL fraction offers optimal conditions for the enzyme-catalyzed reaction due to the high affinity of LCAT for the HDL surface and the high content of apo A-I in the HDL, the enzyme cofactor [21]. Transfer of unesterified cholesterol to the large esterification machinery housed in the HDL fraction is therefore a major driving force for the functioning of the mechanisms destined to promote cholesterol homeostasis in the body. In one of those major mechanisms, the reverse cholesterol transport process, unesterified cholesterol is transferred from the cells

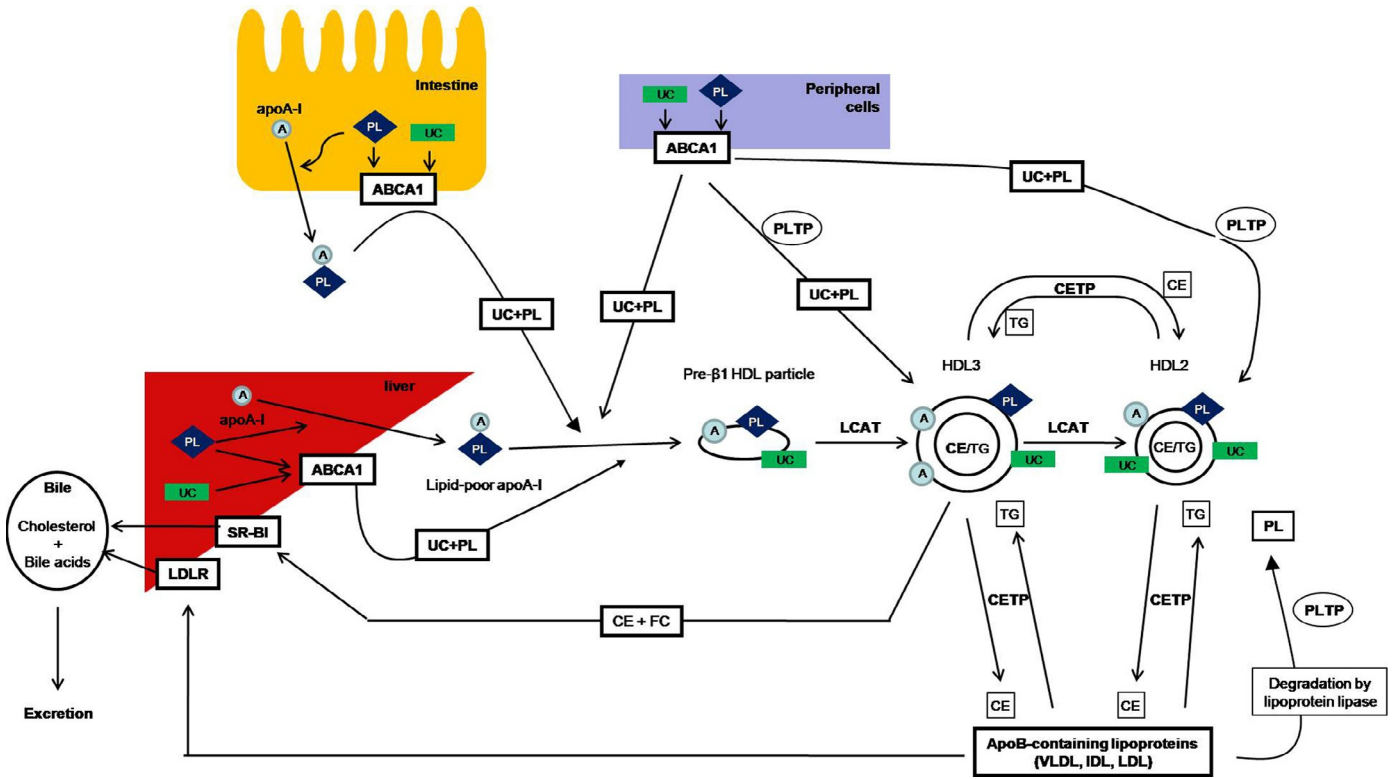

Figure 1.1—CONT'D

of the extrahepatic tissues and vessels to HDL, where it undergoes the esterification reaction.

Remodeling of HDL is also promoted by PLTP that favors the phospholipid efflux to HDL from peripheral cells and from triglyceride-rich lipoproteins, during the degradation of chylomicrons and VLDL by lipoprotein lipase [7].

In the final step of the reverse cholesterol process, cholesteryl esters of HDL are directly shuttled to the liver where the scavenger receptor class B member 1 (SR-BI) takes up the lipoprotein particles and their cholesterol content is excreted in the bile. Alternatively, HDL cholesteryl esters are beforehand transferred to apo B-containing lipoproteins, by the action of CETP, thereby being excreted in the bile after uptake of those lipoproteins by the liver [15].

5. OTHER HDL FUNCTIONS

In addition to the HDL primary functions in cholesterol homeostasis, cholesterol esterification, and reverse cholesterol transport, other increasingly numerous protective functions have been ascribed to this lipoprotein.

HDL antioxidative activity is typically observed as inhibition of LDL oxidation [9]. HDL protects both lipid and protein moieties of LDL and inhibits accumulation of various oxidation products in the LDL fraction, including oxidized phospholipids [22]. HDL is also able to inhibit the

Figure 1.1 *Metabolism of HDL*. Synthesized by the liver and the intestine, apolipoprotein A-I (apo A-I) acquires phospholipid (PL) into the cells. Unesterified cholesterol (UC) and PL efflux from the peripheral cell, via ABCA1, and are taken up by the lipid-poor apo A-I to form pre-β1-HDL particle. The progressive action of lecithin:cholesterol acyl transferase (LCAT) on nascent HDL generates a spectrum of mature, spherical HDL particles: small cholesteryl ester-poor, protein-rich HDL3, and large cholesteryl ester-rich HDL2. These mature HDL particles have a neutral lipid core of cholesteryl ester (CE) and triglyceride (TG) and can acquire additional lipids, via efflux mediated by ABCA1. The phospholipid transfer protein (PLTP) promotes phospholipids efflux to HDL from peripheral cells and from triglycerides-rich lipoprotein, during the degradation of chylomicrons and VLDL by lipoprotein lipase. Unesterified and esterified cholesterol content of mature HDL is shuttled to the liver by an uptake process involving the hepatic scavenger receptor class B member 1 (SR-BI). The cholesterol ester transfer protein (CETP) facilitates the exchange of triglycerides in apoB-containing lipoproteins (VLDL, IDL, and LDL) for cholesteryl ester in HDL. CETP is also responsible to the CE transfer among HDL subclasses. Both the unesterified and esterified cholesterol content of apoB-containing lipoproteins are taken up by the liver, predominantly via the low-density lipoprotein receptor (LDLR). Henceforth, the cholesterol content is excreted in the bile

generation of reactive oxygen species (ROS) *in vitro* [23,24] and *in vivo* [25]. The antioxidative activity of HDL is related to the presence of apo A-I and apo E and enzymes with antioxidative properties that are associated to the HDL particles. It appears that apo A-II is a major component of the antioxidative activity of HDL, which can prevent or delay the LDL oxidation by removing oxidized phospholipids from LDL and from arterial wall cells [26,27]. Apo E possesses antiatherosclerotic activity, which is attributed to its properties in lipid transport [28]. Finally, HDL is able to function as a preventive antioxidant through its ability to bind metal ions, potent catalyzers of LDL oxidation [29].

Major HDL-bound antioxidative enzymes are PON1 and PAF-AH [9]. PON1 is an esterase/lactonase calcium dependent, hydrolyzes a variety of oxidized fatty acids from phospholipids, and prevents the accumulation of oxidized lipids in lipoproteins, mainly in LDL [30–32]. HDL provides the optimal physiological acceptor complex for PON1, stimulating enzyme secretion and stabilizing the peptide [33]. PON1 activity is inversely related to the risk of cardiovascular disease (CVD), independent of all other coronary risk factors, including HDL cholesterol [34].

PAF-AH is a Ca^{2+}-independent enzyme of the phospholipase A2 family involved with the antioxidant and anti-inflammatory functions of HDL [35,36]. PAF-AH has two main functions against the oxidation process: it hydrolyzes PAF, a phospholipid mediator with proinflammatory properties, and degrades oxidized phospholipids [35,37]. PAF accumulates within the atherosclerotic plaques of individuals with CVD, suggesting that this phospholipid mediator may be actively involved in the pathophysiology of atherosclerosis [37,38]. Thus, PAF-AH may function as antiatherogenic enzyme via degradation of phospholipids [38].

The anti-inflammatory activity of HDL is showed by the ability of the lipoprotein to decrease cytokine-induced expression of adhesion molecules on endothelial cells and to inhibit the adhesion of monocytes to those cells [9]. HDL efficiently inhibits *in vitro* the expression of the vascular cell adhesion molecule, intercellular adhesion molecule, and E-selectin, induced by tumor necrosis factor-α (TNF-α), interleukin 1, or endotoxin [39–41].

The anti-inflammatory action of HDL involves the inhibition of TNF-α-stimulated activation of sphingosine kinase and production of sphingosine-1-phosphate (S1P), which induces the expression of adhesion molecules in endothelial cells [42]; TGF-β may function as an important mediator of the anti-inflammatory activity [43]. In addition, HDL attenuates

IL-6 production in endothelial cells exposed to proinflammatory stimuli, such as TNF-α and endotoxin [44]. In fact, this potent anti-inflammatory activity observed *in vitro* was confirmed in a rabbit model of acute arterial inflammation wherein inhibition of adhesion molecule expression and decrease in neutrophil infiltration in the arterial wall was observed after injection of reconstituted HDL [25].

The HDL anti-inflammatory action also involves hydrolysis of oxidized lipids by HDL-associated enzymes (PAF-AH and PON1) and is mechanistically superimposed to the antioxidative activity of HDL [45,46]. Oxidized phospholipids possess potent proinflammatory activities and can trigger arterial inflammation [47]. Inactivation of oxidized lipids by HDL may be associated with decreased expression of adhesion molecules in endothelial cells, which decreases macrophage adhesion to the endothelium [46,48].

HDL may provide additional cardiovascular benefits by antagonizing platelet activity and inactivating the coagulation cascade. It was shown that apo A-I levels correlated with the anticoagulant response to activated protein C/protein S that promotes the inactivation of factor Va [49]. HDL also favors the restoration of the endothelial function [50].

HDL can inhibit the apoptosis of endothelial cells induced by oxidized LDL [51] or TNF-α [52] and decrease the intracellular generation of ROS and levels of apoptotic markers [23]. In addition, HDL contains lysophospholipids that increase the nitric oxide (NO) production, thereby reducing apoptosis of endothelial cells [53–55].

Another important protective function of HDL is the vasodilatory action of the lipoprotein, which is accomplished by three different mechanisms involving endothelial NO synthase (eNOS). First, HDL stimulates the release of NO by endothelial cells, through the mobilization of intracellular Ca^{2+} and phosphorylation of eNOS [56]. Second, not only the release but also the production of endothelial NO is stimulated by HDL, by upregulation of eNOS expression [50]. Finally, binding of HDL to SR-BI receptors plays a role in the activation of eNOS, a process that involves eNOS binding to apo A-I [57].

Other mechanism whereby HDL promotes vasodilation is through stimulation of the production of prostacyclin, which possesses potent vasorelaxing activity [58]. The vasoactive effects of HDL can be mediated by S1P acting via the lysophospholipid receptor S1P3 [54]. S1P may be equally important for mitogenic effects of HDL on endothelial cells and for the inhibitory action of HDL on the migration of vascular smooth muscle cells [59,60].

HDL has also an antithrombotic activity, by inhibiting the factors that promote blood coagulation, including tissue factor, factors X, Va, and VIIIa [9]. This effect may be related to the presence of cardiolipin and phosphatidylethanolamine in the HDL composition, two phospholipids with anticoagulant properties [61]. Stimulation of the production of NO by HDL also accounts for the inhibitory action of HDL on platelet aggregation [62].

HDL also has anti-infectious activity. Through the direct interaction with apo A-I [63], the lipoprotein binds and removes circulating endotoxins for excretion in the bile, decreasing the endotoxin-induced TNF production, and the CD14 expression on monocytes [64,65].

6. PROATHEROGENIC HDL

In some conditions like inflammatory states, diabetes, or metabolic syndrome, apo A-I can be modified by ROS [66]. Apo A-I oxidative modification can hamper the HDL action in cholesterol efflux from the cells by impairing the apo A-I interaction with the ABCA1 complex that pumps out cholesterol from the cell [66]. The antioxidant enzymes associated with HDL can also be inactivated, and oxidized proteins and lipids can accumulate in HDL particles, thus compromising the anti-inflammatory function of the lipoprotein [67,68]. Substitution of SAA for apo A-I in the HDL particle structure can revert HDL function and the lipoprotein becomes proinflammatory and proatherogenic [68–70]. This may occur in conditions associated with high SAA levels in the serum, such as metabolic syndrome, diabetes, and renal chronic disease [68–70].

7. CHOLESTEROL ESTER TRANSFER PROTEIN

CETP, a hydrophobic glycosylated, 476 aa, 75-kDa single polypeptide protein, is secreted primarily by the liver, adipose tissue, enterocytes, and spleen [71] and circulates in the plasma associated principally with HDL [72].

CETP can only promote the net mass transfer of lipids between lipoprotein subclasses that have different cholesteryl ester/triglycerides mass ratios [72]. Therefore, CETP mediates the transfer of cholesteryl esters from HDL to apo B-containing lipoproteins in exchange for triglycerides and the transfer of triglycerides from apo B-containing lipoproteins in exchange for cholesteryl esters [73–75]. CETP also favors the cholesteryl ester transfer among HDL subparticles (pre-β-HDL, -HDL3, and -HDL2) [73].

CETP activity is dependent on both its concentration and ability to interact with lipoproteins. The interaction can be stimulated by free fatty acids generated during hydrolysis of dietary triglycerides [72] or inhibited by specific apolipoproteins, such as apo C-I or apo F [76,77]. Most of the cholesteryl esters in the plasma originates from the LCAT-catalyzed esterification reaction that occurs mainly in the HDL fraction [20]. Triglycerides in the circulation are mostly present in chylomicrons and VLDL before hydrolysis by lipoprotein lipase [72].

As a regulator of cholesterol flux through the reverse cholesterol transport system, CETP may be viewed as potentially having both proatherogenic and antiatherogenic properties. In its proatherogenic action, CETP-mediated cholesteryl ester transfer may decrease the flux of cholesterol through HDL to hepatic SR-BI in the direct reverse cholesterol transport pathway concomitantly increase the mass of cholesterol transported by atherogenic VLDL, IDL, and LDL, from the arterial wall and an increase in atherogenic LDL levels [15,78]. In consequence, it would tend to enhance the cholesterol deposition in peripheral tissues and the arterial wall [78]. CETP also interacts with triglyceride lipases to generate small, dense LDL which is the most atherogenic LDL subclass [79] and HDL [80]. The CETP-mediated reduction in HDL particle size is accompanied by the dissociation of lipid-poor apo A-I from the particle [81,82].

On the other hand, CETP may exert antiatherogenic effects, as it promotes the flux of cholesteryl ester to the liver via indirect reverse cholesterol transport, with hepatic cholesteryl ester uptake predominantly through the antiatherogenic LDL receptor route [73]. Furthermore, CETP contributes to the optimization of LDL particle structure and of apo B100 conformation for high-affinity binding to LDL receptors [83].

The level of CETP activity is determinant in the partition of the cholesteryl ester plasma pool between LDL and HDL. However, excess CETP activity increases the bidirectional transfers between HDL and LDL without importantly changing the distribution of cholesteryl esters between the two lipoprotein plasma fractions [74]. When the level of VLDL is normal, CETP-mediated transfers of HDL cholesteryl esters are directed preferentially to LDL, but when VLDL accumulates in the serum, HDL cholesteryl esters are preferentially transferred by CETP to VLDL particles [84,188].

The crystal structure of CETP by X-ray crystallography consists of an elongated boomerang-shaped asymmetric conformation with N- and C-terminal β-barrels, a central β-sheet between the two β-barrels, and a

hydrophobic central cavity, with the curvature of the concave surface likely fitting to the convex curvature of the lipoprotein surface [85]. This cavity is filled by two cholesteryl ester molecules and communicates with two openings near the central β-sheet domain. These pores, which are occupied by two phospholipid molecules, could be gates for the interaction of the central cavity with the aqueous environment or lipoproteins [85].

In respect to the mechanisms through which CETP acts in lipid exchanges, some hypotheses have been proposed. CETP would transport cholesteryl esters between donor and acceptor lipoproteins, through the aqueous phase [86]. Alternatively, in a sort of tunnel mechanism, CETP would bridge two lipoproteins; HDL binds on N-terminal and LDL or VLDL binds on C-terminal end. A transient ternary complex is formed, with neutral lipids flowing from the donor to acceptor lipoprotein through the CETP molecule [87,88]. In a modified tunnel hypothesis, lipids would be tunneled in a CETP dimer [72].

The decrease in HDL levels and antiatherogenic functions and the increase in proatherogenic factors that occur in metabolic syndrome and in type 2 diabetes mellitus (T2DM) patients coexists with high CETP concentration in the plasma [89,90]. On the other hand, treatment with pioglitazone decreased the hepatic triglyceride content and increased the HDL cholesterol levels while simultaneously decreased CETP [91]. In normal-weight subjects, there is an inverse correlation between CETP level and visceral fat gain and CETP level and body mass index [92].

7.1. CETP inhibitors

Increased CETP plasma levels can reduce HDL cholesterol [189], and CETP deficiency is associated with elevated HDL cholesterol [190]. Aiming to expand the HDL fraction, several chemical CETP inhibitors have been developed, including torcetrapib (Pfizer, USA), dalcetrapib (Roche, Switzerland), anacetrapib (Merck, USA), and evacetrapib (Lilly, USA). The molecular mechanisms of CETP-mediated lipid transfers among lipoproteins are still largely unknown.

Torcetrapib, a 3,5-bis(trifluoromethyl)phenyl derivative, is a potent inhibitor of CETP activity, which enhances the association between CETP and HDL to form a complex that inhibits the transfer of lipids between HDL and other lipoproteins [93]. Torcetrapib was able to increase HDL cholesterol level by 72% and to decrease LDL cholesterol by 25% [94]. However, despite the favorable lipid changes, torcetrapib failed to diminish maximum

carotid intima-media thickening in patients with familial hypercholesterolemia [95] and in mixed dyslipidemia [96]. In 2006, the ILLUMINATE study was prematurely finished due to the adverse effects of the drug, excess of deaths, and CVD [94].

Further analysis of the trial data revealed other undesirable effects of torcetrapib, including increased blood pressure, sodium, bicarbonate, and aldosterone levels, and decreased potassium levels [94,97]. Hypertension resulted from increased production of adrenal steroids, such as aldosterone and cortisol. It is currently understood that the adverse events caused by torcetrapib are molecular-specific and do not relate to the mechanism of CETP inhibition [15]. Studies with animal models demonstrated increase in the aortic wall expression of endothelin in response to torcetrapib administration [98]. Together with data of human adrenal cell assays [99], these results suggest that these off-target effects are related to the stimulation of aldosterone synthesis by torcetrapib via pathways independent of CETP inhibition [15]. Structure–activity investigations have provided further evidence that the hypertensive effects of torcetrapib are unrelated to CETP inhibition [100].

Dalcetrapib, a benzenethiol derivative, was the first small molecule showed to inhibit CETP and to possess an antiatherogenic effect *in vivo* [101]. It binds CETP irreversibly but, in contrast to torcetrapib, dalcetrapib does not appear to induce the formation of a CETP–HDL complex at therapeutic plasma concentrations [102]. Dalcetrapib is considerably less potent than torcetrapib as HDL raiser. In healthy individuals, daily treatment with 600 mg of dalcetrapib increased HDL levels by 23%, after 4 weeks [103] and by 28% in type II patients receiving pravastatin, with decrease LDL cholesterol levels by 7% [104]. After 24 weeks, dalcetrapib (900 mg/day) was able to raise HDL cholesterol levels by 33% in patients receiving atorvastatin, but levels of LDL cholesterol did not alter [105]. Despite the effects on HDL cholesterol, some encouraging imaging observations and the warrant from the safety data, dalcetrapib failed to achieve reduction of cardiovascular events and the clinical trials were stopped in 2012.

Anacetrapib is other 3,5-bis(trifluoromethyl) phenyl derivatives used as CETP inhibitor, by forming a tight reversible bond with this protein [106]. Anacetrapib inhibits the transfer of cholesteryl esters from HDL to LDL and from HDL3 to HDL2 [106]. In both normolipidemic individuals and dyslipidemic patients treated with atorvastatin, anacetrapib (300 mg/day) was able to increase the HDL cholesterol levels by 130%, apo A-I by 47%, and decrease the LDL cholesterol levels by 40% [107,108]. Anacetrapib treatment does not increase blood pressure or aldosterone synthesis

[107,108]. The safety of the drug was proved in patients with coronary heart disease or equivalent conditions [109,110].

Anacetrapib can improve not only HDL cholesterol but also measures of HDL functionality. HDL sampled from anacetrapib-treated patients promoted greater cholesterol efflux from foam cells in culture, independent of HDL cholesterol levels, while maintaining the anti-inflammatory activity of HDL [111].

The ongoing Phase III REVEAL study was designed to test whether anacetrapib will reduce the incidence of major coronary events (coronary mortality, myocardial infarction, and coronary revascularization) in 30,000 patients with established CVD, undergoing statin therapy and is expected to be completed by 2017 [112,113].

Evacetrapib, the most recent benzazepine-based CETP inhibitor in clinical development, is a novel benzazepine compound with selective and potent CETP inhibitory activity [114]. In monotherapy, this drug produced dose-dependent increase in HDL cholesterol level ranging from 54% to 130% and decrease in LDL cholesterol ranging from 14% to 36% [115]. In combination with the statin therapy, evacetrapib also raised HDL cholesterol and decreased LDL cholesterol levels (83% and 13%, respectively). In that trial, no alterations in blood pressure, aldosterone levels, or mineralocorticois were found [115]. Those findings were determinant for the performance of larger clinical trials to evaluate evacetrapib that are currently on the march.

8. PHOSPHOLIPID TRANSFER PROTEIN

PLTP is a hydrophobic, glycosylated, single polypeptide, 476 aa, 80 kDa [116] that belongs to the lipopolysaccharide (LPS) binding/lipid transfer gene family that includes the LPS-binding protein, the neutrophil bactericidal permeability increasing protein, and CETP [117]. All are able to bind and transfer LPS and phospholipids [117]. PLTP has a 25% amino acid homology with CETP [116] and a boomerang-shaped molecule is predicted by three-dimensional structural modeling, with two domains and a hydrophobic pocket in each domain for binding the acyl chains of phospholipids [118].

PLTP is present in the plasmin both the active and the inactive forms, the latter at a 50–90%proportion [119–121]. The two forms are associated with macromolecular complexes of different size and composition [119,120]. Complexes with active PLTP, which is associated with apo E, are smaller

with 7.6–12.0 nm diameter, whereas complexes with inactive PLTP, which is associated with apo A-I, are larger with 12–17 nm diameter [119,121,122]. Since lipids account for only 3% of the PLTP complexes, it is likely that protein–protein interactions, rather than protein–lipid interactions, drive the assembly of PLTP complexes [123]. Those observations suggest that PLTP is secreted as a high-activity complex, and during phospholipid transfer from triglyceride-rich lipoproteins to HDL, it forms complex with apo A-I and loses phospholipid transfer activity [122].

PLTP is unable to transfer neutral lipids between LDL and HDL, but similar to CETP, PLTP promotes net mass transfer of phospholipids from phospholipid vesicles or degraded VLDL particles into HDL [124,125]. Indeed, PLTP facilitates the transfer of a spectrum of different amphipathic compounds, such as diacylglycerols, phosphatidic acid, sphingomyelin, phosphatidylcholine, phosphatidylglycerol, cerebroside and phosphatidyl ethanolamine, free cholesterol, lipopolysaccharide, and vitamin E [7,117,126].

PLTP is essential for the transfer of excess surface lipids from triglyceride-rich lipoproteins to HDL, during lipolysis of chylomicrons and VLDL by lipoprotein lipase [124]. In addition, PLTP may contribute to the remodeling of HDL particles. *In vitro* studies suggest that alteration of lipid composition of HDL by phospholipid transfer may result in destabilization of its apolipoproteins, loss of apo A-I, and consequent fusion of lipoprotein particles [123,127].

PLTP favors the generation of large α-HDL and of pre-β-HDL particles. Transfer of vitamin E mediated by PLTP contributes to diminish the vitamin E content of circulating lipoproteins and to increase their oxidability [126], an effect that is potentially atherogenic.

PLTP facilitates the efflux of cholesterol from peripheral cells through multiple mechanisms. PLTP promotes cell-surface binding and remodeling of HDL, which improves the ability of HDL to remove cholesterol and phospholipids from cells [128]. The continuous generation of efficient acceptors of cellular cholesterol mediated by PLTP is one of the important pathways that PLTP facilitates reverse cholesterol transport. During the PLTP-mediated conversion process, small apo A-I particles and large fused HDL particles are generated, which are efficient cholesterol acceptors [129]. Indeed, pre-β-HDL precursors could be also formed by PLTP action as it transfers apo A-I/phospholipid complexes from chylomicrons during lipolysis [123]. It is also known that PLTP can bind directly to ABCA1, leading to solubilization of the surface lipids and their removal from the cells by PLTP.

Subsequently, surface lipids are shuttled to lipoprotein acceptor particles [130]. Alternatively, a PLTP–lipoprotein complex can interact directly with the cell-surface lipid domains generated by ABCA1 and thereby remove the lipids from the cell surface [130].

Some studies showed that there are metabolic relationship between the lipoproteins and plasma PLTP activity, mass, and specific activity. In healthy subjects, triglyceride and HDL cholesterol levels and HDL particle size are significant independent modulators of plasma PLTP activity, mass, and specific activity, respectively [131]. Plasma PLTP concentration was positively associated with HDL cholesterol, specifically with HDL2-cholesterol, large HDL particles, and mean HDL particle size, but was inversely associated with triglyceride and BMI [131].

Some studies have shown that plasma PLTP activity is positively associated with total cholesterol, triglycerides, apo B, VLDL, and IDL [131,132]. Indeed, there is evidence that gender and obesity-related factors, such as hormones and hepatic triglyceride lipase, can mediate these associations [133–135].

PLTP expression is increased in different diseases associated with increasing risk of coronary artery disease, such as obesity, insulin resistance, and type I and type II diabetes [136–138]. In a cross-sectional study, it was reported that serum PLTP activity is increased in CVD patients [139]. Moreover, PLTP activity is positively correlated with left ventricular systolic dysfunction [140] and low HDL levels [141]. In contrast, it was also reported that lower PLTP activity is a risk factor for peripheral atherosclerosis [139,142]. It was documented that PLTP concentration is lower in subjects with hypoalphalipoproteinemia associated with severe conditions such as Tangier disease, LCAT deficiency, apo A-I deficiency, and familial HDL deficiency [143]. Meanwhile, PLTP is higher in patients with hyperalphalipoproteinemia associated with CETP deficiency [143]. Some observations in transgenic mice corroborated those data; in human apo A-I transgenic background mice, overexpression of PLTP results in an increase in HDL cholesterol and apo A-I as well as an increase in pre-β-HDL [144]. Reduced plasma PLTP activity in knockout mice results in decreased HDL lipid and apolipoprotein content, which supports the importance of the transfer of surface components of triglyceride-rich lipoproteins in the maintenance of HDL levels [125].

PLTP has also vitamin E transfer activity, which is determinant in the bioavailability of this vitamin. Transfer of α-tocopherol mediated by PLTP can modulate oxidation of lipoproteins and tissues [144,145]. Vitamin

E-enriched LDL from PLTP-deficient mice is resistant to oxidation and also is much less likely to induce monocyte chemotactic activity [126,146]. Moreover, PLTP overexpression in apo E knockout mice leads to decrease in the vitamin E content of LDL and increase in LDL oxidation [147].

PLTP can also modulate phosphatidylserine externalization in erythrocytes, which can have an impact on the risk of thrombus formation [148]. Indeed, *in vitro* assay data showed that PLTP may play a protective role in thrombosis [149,150].

9. CETP AND PLTP IN CVD

Whether CETP and PLTP concentrations and activities are related with the incidence of CVD is still a matter of controversy. This fact is presumably due to the diversity of experimental conditions and the presence of confounding factors, such as low HDL cholesterol, elevated plasma triglycerides, and LDL/HDL ratio [151,152]. With respect to CETP, some studies of CETP polymorphism have showed that the CETP activity is inversely related with HDL cholesterol and directly with the degree of atherosclerosis [153]. In contrast, there are some large population studies that showed that low levels of CETP concentration or activity were associated with higher levels of plasma HDL cholesterol but not with lower incidence of CVD [154–156].

Clinical evidence about the role of PLTP in the CVD occurrence is more limited than for CETP. Some clinical studies have suggested that higher levels of PLTP activity, independently of plasma HDL cholesterol increased, are associated with an increase in cardiovascular events [138,157]. On the other hand, other few studies showed that a high rate of atherosclerosis is related with the reduction of the PLTP activity [123]. In addition, a large analysis has found, based on several single nucleotide polymorphisms of PLTP, that development risk of CVD was lower with a lower overall PLTP gene score [157]. In a most recent report, a prospective data from the Framingham Heart Study, low CETP activity in conjunction with high PLTP activity was related with the development of CVD in the male but not in the female gender, independent of the classical risk factors of the disease [158].

According to Albers *et al.*, the contribution of CETP and PLTP to atherosclerosis development appears to depend on the genetic background and the physiological context, and requires the generation of a more atherogenic lipoprotein phenotype [123].

10. SIMULTANEOUS TRANSFER OF LIPIDS TO HDL

It has increasingly being perceived that although a strong correlation between HDL cholesterol and incidence of CVD occurs in most tested populations, HDL cholesterol is not sufficient to account for the wide variety of atheroprotective functions of the lipoprotein. It is conceivable that higher concentrations of HDL particles in the circulation may increase the esterification of the plasma cholesterol pool and the extraction of cholesterol from peripheral cells [20]. Additionally, higher HDL concentrations offer a larger harboring ground for the several proteins that exert the variety of protective functions related with the lipoprotein [9]. Nonetheless, many other intervening factors may change the HDL cholesterol concentration paradigm. A classical example is the population of carriers of apo A-I_{Milano} mutation, a natural variant of apo A-I characterized by a single amino acid substitution in 173, where an arginine is replaced by a cysteine [159]. Those subjects have low levels of plasma HDL cholesterol and apo A-I with moderate hypertriglyceridemia [160]; despite this proatherogenic phenotype, A-I_{Milano} carriers have reduced cardiovascular risk [161,162]. *In vitro* studies showed that serum from the apo A-I_{Milano} carriers and from transgenic mice expressing the apo A-I_{Milano} variant is as efficient as serum from control subjects in promoting cell cholesterol efflux, despite the large reduction in serum apo A-I and HDL concentrations [163–166]. On the other hand, Khera *et al.* [167] showed that, in patients with subclinical atherosclerosis and obstructive coronary artery disease, the cholesterol efflux from cultivated murine macrophages was decreased, independently of the levels of HDL cholesterol and apo A-I. Those findings support that the cholesterol efflux capacity of HDL is not explained solely by the concentration of HDL cholesterol or apo A-I.

The phenomena related to lipid transfers among lipoprotein classes have been extensively explored, and CETP and PLTP actions have been examined in several clinical conditions, despite many unsolved questions regarding the participation in atherogenesis and the validity of CETP inhibition as a therapeutic target. However, a more comprehensive picture of the lipid exchanges involving HDL is lacking and may be of great interest. HDL is the main cholesterol esterification site in the plasma, and is the main driver of reverse cholesterol transport, which is essentially a lipid transfer phenomenon [21]. In addition, lipid transfers chiefly affect the HDL composition, structure, and size and, consequently, the HDL subclass profile in the plasma

compartment [82,131]. As anchoring of proteins to HDL particles depends on HDL composition and size [9], it is conceivable that the cast of those proteins that are responsible for many of the HDL functions are subjected to changes related with transfer of lipids to the lipoprotein.

In our view, an approach for the transfer of lipids to HDL was lacking aiming to systematically study the phenomenon in large populations. As a requirement, the new approach should be practical and standardized enough to be suitable as a routine procedure in the clinical biochemistry laboratory. An integrated assessment of the main lipid species involved in the transfers would be also of prior interest, since the exchange of a given lipid species often occurs in conjunction with others and all those lipid shifts influence HDL metabolism and function. Aiming to achieve these goals, we recently developed an *in vitro* assay that fulfills those requirements and is currently under examination regarding the clinical relationships derived from the measurement of the transfer parameters [168].

The assay consists in the incubation of a lipidic donor nanoemulsion with whole plasma (Fig. 1.2). The nanoemulsion is prepared from mixtures of phosphatidylcholine and cholesteryl oleate, with small amounts of triolein and unesterified cholesterol [168]. For the execution of the assay, to avoid time-consuming lipid separation before radioactive counting, two sets of labeled nanoemulsions are manufactured, for example, one with ^{3}H-triolein and ^{14}C-unesterified cholesterol and the other with ^{3}H-cholesteryl oleate and ^{14}C-phosphatidyl choline. After incubation in a shaking bath for 1 h at 37 °C, the apo B-containing lipoproteins and the nanoemulsion are precipitated by addition of dextran sulfate/$MgCl_2$ as precipitation reagent. The radioactivity of the supernatant is then measured in liquid scintillation analyzer. Data are calculated as % of the total incubated radioactivity [168].

In the sampled plasma, most of the components that physiologically influence the transfers are present, such as CETP, PLTP and LCAT, and the other lipoprotein classes that compete with HDL for the reception of the donor nanoemulsion lipids. Nonetheless, other factors such as lipoprotein lipase and hepatic lipase that can act on the transfer *in vivo* are not at play. The data generated by the assay are dependent of the action of transfer proteins and LCAT, composition and concentration of HDL, and of the apo B-containing lipoproteins that compete with HDL for reception of the nanoemulsion lipids, among other factors [168]. The transfer assay method was validated for precision and reproducibility. Plasma samples used in the transfer assay can be stored at −80 °C for about 1 year, without changes in the results [168].

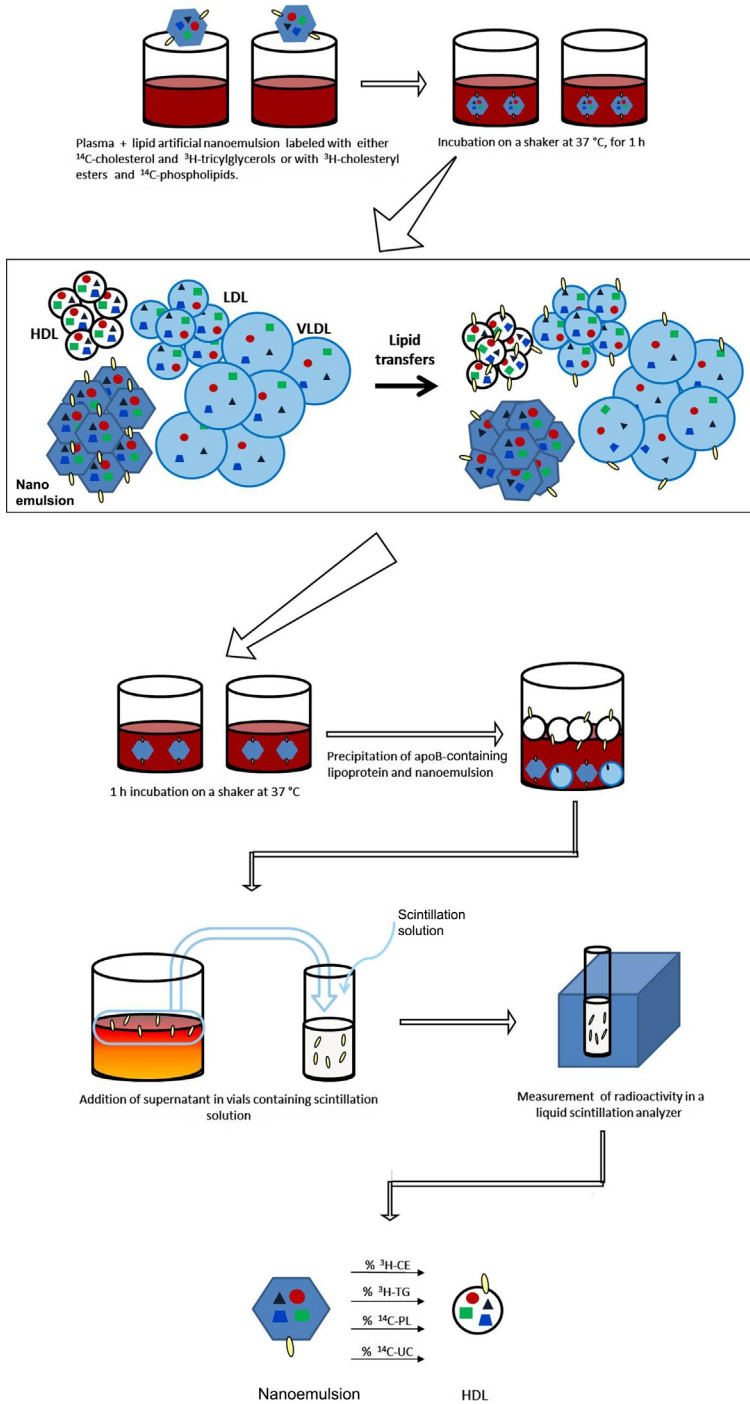

Figure 1.2 *In vitro lipid transfer assay*. The assay is performed using a donor nanoemulsion that resembles the LDL lipid structure and prepared according to Ref. [168]. (*Continued*)

The aim of developing the novel method was to disclose changes in the lipid transfer profile that could provide new markers of disease, to evaluate drug, nutritional, and lifestyle effects on HDL function, or to eventually help to devise new therapeutic or diagnostic tools.

In all the subject groups studied to date, the transfer of phospholipids was markedly greater than that of the other three lipids, in the order of 18–25%. Unesterified cholesterol transfers to HDL at 5–9% rate, whereas esterified cholesterol and triglycerides transfers display the lowest transfer rates, at 3–7% range. It is conceivable that lipids located at the lipoprotein or nanoparticle surface, phospholipids and unesterified cholesterol, are more prone to be dislocated from the particles, whereas lipids that constitute the particle core, triglycerides, and cholesteryl esters are less liable for shifts from the lipoprotein structure [168]. On the other hand, penetration of the lipids dissociated from the artificial nanoemulsion into the core of HDL is presumably more difficult than positioning in the lipoprotein surface layer.

It is noteworthy to point out that transfer of lipids is bidirectional [74]. As in this assay only the nanoemulsion-to-HDL direction is measured, it is not possible to ascertain that greater or smaller transfers of a given lipid species from the nanoemulsion to HDL imply in trends for enrichment or impoverishment of HDL with the lipid.

Relative to CETP, which is a crucial driving force for triglyceride and cholesteryl ester transfer, no correlation was found in those normal, healthy individuals, between CETP concentration in the plasma and the transfer of all four lipids (Maranhão R.C. *et al.*, unpublished data). Probably, equilibrium of the lipid transfers was already attained with the lower CETP levels and higher levels of the protein did not further increase the transfers. The interindividual variations can thus be ascribed to the other factors intervening in the transfer processes. However, in 80 patients with type 2 diabetes, CETP concentration positively correlated with the transfers of cholesteryl ester, triglycerides, and unesterified cholesterol (Maranhão R.C. *et al.*, unpublished data). It is well documented that CETP is increased in

Figure 1.2—CONT'D Two sets of lipid nanoemulsions are prepared, one with ^{3}H-triolein and ^{14}C-unesterified cholesterol and the other with ^{3}H-cholesteryl oleate and ^{14}C-phosphatidyl choline. Plasma is incubated with the radioactively labeled nanoemulsion, for 1 h at 37 °C on shaker. After the incubation, the apo B-containing lipoproteins and the nanoemulsion are chemically precipitated. The radioactivity of the supernatant that contains the HDL fraction is measured in a liquid scintillation analyzer. Data are expressed as % of the total incubated radioactivity measured in the supernatant.

diabetes [89], and our findings illustrate the complexity of the interplay of the many factors which affect the lipid transfer phenomena.

Although in normolipidemic healthy subjects there is a correlation between HDL cholesterol concentration and all lipid transfers to HDL, in the studies on diseased states or interventions, the transfers were frequently lower or increased in the cases compared to controls, despite the HDL cholesterol levels being not altered accordingly.

In the results exposed in Table 1.1, it is appealing that the decreased transfer of unesterified cholesterol to HDL is the marker of the presence of coronary artery disease. This was found in both studies of nondiabetic coronary artery disease patients, of older [169] and precocious coronary artery disease [171], and also in the study in which type 2 diabetes patients with coronary artery disease were compared to diabetic patients without coronary artery disease (Maranhão R.C. *et al.*, unpublished data). It is worthwhile to point out that in nondiabetic coronary artery disease, the triglyceride transfer was also diminished compared to controls [169]. However, in diabetics with coronary artery disease, triglyceride transfer was not different from diabetics without coronary artery disease (Maranhão R.C. *et al.*, unpublished data).

Diminution of free cholesterol transfer to HDL can lead to reduction of the esterification process and impair the cholesterol reverse transport. Sequestration of cholesterol into the lipoprotein core by esterification is also protective because excess unesterified cholesterol in the lipoprotein surface could diffuse into the surrounding aqueous medium and eventually precipitate in the artery [185]. This was suggested in a study in which the plasma kinetics of the esterified and nonesterified cholesterol components of a nanoemulsion that resemble LDL lipid structure was determined in coronary artery disease and noncoronary artery disease subjects [185]. In the former group, the unesterified moiety tended to dissociate from the nanoemulsion particles, with faster removal from the circulation than the particles marked by the cholesteryl ester component. Indeed, in coronary artery disease patients that were being submitted to heart revascularization surgery, the content in the artery of radioactive unesterified cholesterol was greater than that of the esterified cholesterol [186], suggesting deposition of the unesterified cholesterol molecules that was independent from the arterial uptake of the nanoemulsion particles. Enrichment of the artery with unesterified cholesterol from the plasma, dissociated from the lipoproteins, could disturb the antiatherosclerosis functions of the endothelial pavement of the artery.

Table 1.1 Comparative transfer of four radioactively labeled lipids from a donor nanoemulsion to HDL according to gender, age, menopausal status, and in different diseases, statin use, exercise training, and dieting

Condition/disease	HDL cholesterol concentration	Lipid transfer to HDL				Subject characteristics	Reference
		Unesterified cholesterol	Triglycerides	Cholesteryl ester	Phospholipid		
Men vs. women	↔	↓	↔	↓	↔	Men: 56 ± 12 y; $n = 38$ Women: 59 ± 8 y; $n = 40$	Unpublished data
Elderly vs. young	↑	↔	↔	↑	↑	Elderly: 75 ± 8 y; 11 M, 14 F Young: 25 ± 5 y; 10 M, 15 F	[169]
Postmenopausal vs. premenopausal	↔	↔	↔	↔	↔	Postmenopausal: 48 ± 3 y;18 F Premenopausal: 45 ± 3 y; 22 F	[170]
CAD elderly vs. healthy elderly	↓	↓	↓	↓	↔	CAD elderly: 74 ± 5 y; 5 M, 6 F Health elderly: 75 ± 8 y; 11 M, 14 F	[169]
Precocious CAD vs. non-CAD	↔	↓	↓	↑	↔	Early CAD: 47 ± 1 y; 10 M;	[171]

Continued

Table 1.1 Comparative transfer of four radioactively labeled lipids from a donor nanoemulsion to HDL according to gender, age, menopausal status, and in different diseases, statin use, exercise training, and dieting—cont'd

Condition/disease	HDL cholesterol concentration	Lipid transfer to HDL				Subject characteristics	Reference
		Unesterified cholesterol	Triglycerides	Cholesteryl ester	Phospholipid		
						20 F Non-CAD: 47 ± 2 y; 10 M; 20 F	
CAD under statin treatment vs. CAD without statin treatment	↔	↓	↓	↓	↓	CAD + statin: 58 ± 7 y; $n = 25$ CAD without statin: 57 ± 6 y; $n = 27$	[168]
Type 2 diabetes mellitus with CAD vs. Type 2 diabetes mellitus	↔	↓	↔	↓	↔	T2DM + DAC: 63 ± 8 y; 46 M; 39 F T2DM: 63 ± 9 y; 36 M; 44 F	Unpublished data
Glucose intolerance vs. control	↔	↔	↔	↔	↔	GI: 56 ± 6 y; 5 M, 9 F Control: 53 ± 6 y; 7 M, 8 F	[172]

Metabolic syndrome vs. control	↓	↓	↓	↓	↔	MetS: 50 ± 10 y; 10 M, 10 F Control: 45 ± 7 y; 6 M, 4 F	[173]
Type 2 diabetes mellitus vs. control	↔	↔	↑	↑	↔	T2DM: 59 ± 5 y; 7 M, 8 F Control: 55 ± 6 y; 5 M, 6 F	[174]
Type 1 diabetes mellitus vs. control	↔	↔	↔	↔	↔	T1DM: 26 ± 7 y; 16 M Control: 28 ± 6 y; 15 M	[175]
Familial hypercholesteromia vs. normolipidemia	↔	↓	↑	↔	↑	FH: 39 ± 15 y; 26 M, 45 F NL: 38 ± 11 y; 27 M, 39 F	[176]
Polycystic ovary syndrome vs. control	↔	↔	↔	↔	↔	PCOS: 23 ± 3 y; 18 F Control: 27 ± 5 y; 10 F	[177]
PCOS obese vs. PCOS nonobese	↔	↔	↔	↔	↔	Obese: 26 ± 5 y; 15 F Nonobese: 23 ± 3 y; 8 F	[177]

Continued

Table 1.1 Comparative transfer of four radioactively labeled lipids from a donor nanoemulsion to HDL according to gender, age, menopausal status, and in different diseases, statin use, exercise training, and dieting—cont'd

Condition/disease	HDL cholesterol concentration	Lipid transfer to HDL				Subject characteristics	Reference
		Unesterified cholesterol	Triglycerides	Cholesteryl ester	Phospholipid		
Subclinical hypothyroidism vs. control	↔	↔	↓	↔	↓	SCH: 46 ± 4 y; 12 F Control: 40 ± 8 y; 10 F	[178]
SCH, after 6 weeks of T4 treatment vs. before treatment	↔	↔	↔	↔	↑	After T4 treatment: 46 ± 4 y; 12 F Before T4 treatment: 47 ± 4 y; 12 F	[178]
Heart transplantation vs. control	↓	↓	↔	↓	↔	Transplantation: 51 ± 11 y; 16 M, 4 F Period elapsed: 7 mo–14 y Control: 45 ± 9 y, 16 M, 4 F	[179]
HIV^+ vs. control	↔	↓	↓	↔	↑	HIV^+: 37 ± 8 y; 22 M, 26 F Control: 38 ± 9 y; 16 M, 29 F	[180]

Vegan vs. omnivore	↔	↔	↔	↓	↔	Vegan: 35 ± 10 y; 11 M, 10 F Omnivore: 37 ± 9 y; 17 M, 12 F	[181]
No-trans margarine vs. butter consumption	↔	↓	↓	↔	↔	Margarine: 47 ± 19 y; 6 M, 10 F Butter: 49 ± 12 y; 7 M, 11 F	[182]
Resistance training vs. sedentary	↔	↔	↔	↔	↔	Resistance Training: 25 ± 5 y; 15 M Regular training program for ~2.5 y Sedentary: 28 ± 7 y; 15 M	[183]
Metabolic syndrome, after vs. before training load	↔	↑	↔	↑	↔	MetS: 50 ± 10 y; 10 M, 10 F Short-term training on a bicycle ergometer	[173]

Continued

Table 1.1 Comparative transfer of four radioactively labeled lipids from a donor nanoemulsion to HDL according to gender, age, menopausal status, and in different diseases, statin use, exercise training, and dieting—cont'd

Condition/disease	HDL cholesterol concentration	Lipid transfer to HDL				Subject characteristics	Reference
		Unesterified cholesterol	Triglycerides	Cholesteryl ester	Phospholipid		
Marathon runner vs. sedentary	↑	↑	↑	↔	↑	Runner: 38 ± 7 y; 14 M Sedentary: 37 ± 4 y; 28 M	[184]
Marathon runner, immediately after a marathon vs. at rest period	↔	↓	↓	↓	↓	38 ± 7 y; 14 M	[184]
Marathon runner, 72 h vs. immediately after a marathon	↔	↑	↑	↔	↑	38 ± 7 y; 14 M	[184]

HDL cholesterol concentration in the plasma is also compared.
↑, ↓, or ↔: increase, decrease, or equivalent in first condition to the second condition.
CAD, coronary artery disease; FH, familial hypercholesterolemia; GI, glucose intolerance; MetS, metabolic syndrome; NL, normolipidemia; PCOS, polycystic ovary syndrome; SCH, subclinical hypothyroidism; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; M, males; F, females; y, years; mo, months.

It is difficult to figure out the mechanisms that lead to diminished transfer of unesterified cholesterol from the nanoemulsion to the HDL fraction in coronary artery disease patients. It is possible that in coronary artery disease, the HDL fraction is saturated with unesterified cholesterol, lacking the gradient to receive larger amounts of the lipid. Other compositional factors relative to HDL or the apo B-containing lipoproteins, transfer proteins, and enzymes may also have had a role in this coronary artery disease-associated outcome.

In males, who develop coronary artery disease earlier than females, the transfer of unesterified cholesterol to HDL was decreased (Maranhão *et al.*, unpublished data). Menopause, as observed in pre- and postmenopausal women of about the same age, had no impact upon lipid transfers [170]. Low unesterified cholesterol transfer was also found in patients with heart transplantation [179]; those patients are prone to develop graft coronary disease, a form of accelerated atherosclerosis which is the main cause of morbidity and mortality after the first year from the surgery. In familial hypercholesterolemic patients, a condition that also predisposes to premature coronary artery disease, the transfer of unesterified cholesterol was diminished, while the transfers of triglycerides and phospholipids were increased in comparison to normolipidemic subjects [176]. In those patients, the transfer of cholesteryl ester was not altered [176]. Therefore, not only in patients with documented coronary artery disease but also in conditions associated with coronary artery disease development, there was a consistent trend for reduced transfer of unesterified cholesterol in the HDL plasma fraction. Moreover, it was shown that HIV-infected patients had diminished unesterified cholesterol transfer comparing to healthy individuals [180]. HIV-infected patients have also a greater burden of subclinical and clinical atherosclerotic disease compared to the general population [187].

The diminished triglycerides transfer in nondiabetic subjects is difficult to interpret in terms of CETP action or HDL pathophysiology, but alterations in cholesteryl ester and triglycerides content result in HDL instability and decrease in the concentration of the lipoprotein. In older subjects with coronary artery disease, HDL lowering associated with the diminished triglycerides indeed occurred [169]. In T2DM, the fact that both cholesteryl ester and triglyceride transfer to HDL were increased is conceivably consequent to the increased activity of CETP in this disease [174]. In patients with metabolic syndrome, cholesteryl ester and triglyceride transfers that are increased in T2DM are decreased [173]. Patients with glucose intolerance without metabolic syndrome showed no alterations in the profile of lipid transfers

[172]. Interestingly, in type 1 diabetes mellitus, where alterations in lipid profile such as high triglycerides and low HDL cholesterol that are typical of type T2DM are not prominent, no abnormality in lipid transfers occurred [175]. All those findings await for further investigational effort to ascertain the solidity and significance of the association of transfers and those metabolic diseases.

An important outcome of the studies listed in Table 1.1 is that lipid transfers, as assayed *in vitro*, are also modifiable factors. Statin use depressed the transfer of all lipids in coronary artery disease patients [168]. Compared to sedentary subjects, marathon practitioners had increases in the transfer of all lipids but cholesteryl esters [184]. However, immediately after they run the marathon, the lipid transfers were depressed, recovering the pre-marathon levels after 72 h rest [184]. In contrast, resistance regular (four to five times per week) exercise training for more than 2 years had no effect in lipid transfers [183]. Compared to omnivore, vegan diet followers had lower cholesteryl ester transfer [181]. Substitution of margarine for butter in spreads reduced the transfer of unesterified cholesterol and triglycerides [182].

Lipid transfers to HDL were also examined in patients with polycystic ovary syndrome and subclinical hypothyroidism. In the former group, no differences from the controls were found, regardless of they were obese or not [177]. In subclinical hypothyroidism, the triglyceride and phospholipid transfers were diminished [178]. Those were the only plasma lipid parameters found in those patients that otherwise had normal plasma LDL and HDL cholesterol together with normal removal from the plasma of triglyceride-rich lipoproteins. After they were treated for 6 weeks with hormonal reposition, the transfer of phospholipids became normal, whereas the triglyceride transfer remained low [178].

11. CONCLUSION

The novel *in vitro* assay on the lipid transfers to HDL may independently predict the presence of CVD, and prospective studies are needed to confirm its importance as a CVD risk factor. It is challenging that the lipid transfers are altered in metabolic, inflammatory, and infectious diseases and can be modifiable by exercise training, dietary maneuvers, and drug treatments. To expand the capabilities of the method and the mechanisms behind the results, the overall relationships with HDL metabolism, composition, and subfraction profile should be explored, as well with all possible factors involved in lipid transfers.

ACKNOWLEDGMENTS

The studies on the use of the *in vivo* transfers of lipids to HDL were supported by the Research Support Foundation of the State of São Paulo (FAPESP, São Paulo, Brazil). Dr. Maranhão is a Research Awardee of the Federal Council for Scientific and Technological Development (CNPq, Brasilia, Brazil). The authors are thankful to Fernanda O. Martins, MSc, for her help in reviewing the manuscript.

REFERENCES

[1] J.S. Hagerman, R.G. Gould, The in vitro interchange of cholesterol between plasma and red cells, Proc. Soc. Exp. Biol. Med. 78 (1951) 329–332.
[2] C.S. Rehnborg, A.V. Nichols, The fate of cholesteryl esters in human serum incubated in vitro at 38 degrees, Biochim. Biophys. Acta 84 (1964) 596–603.
[3] D.B. Zilversmit, L.B. Hughes, J. Balmer, Stimulation of cholesterol ester exchange by lipoprotein-free rabbit plasma, Biochim. Biophys. Acta 409 (1975) 393–398.
[4] N.M. Pattnaik, A. Montes, L.B. Hughes, D.B. Zilversmit, Cholesteryl ester exchange protein in human plasma isolation and characterization, Biochim. Biophys. Acta 530 (1978) 428–438.
[5] J. Ihm, J.A. Harmony, J. Ellsworth, R.L. Jackson, Simultaneous transfer of cholesteryl ester and phospholipid by protein(s) isolated from human lipoprotein-free plasma, Biochem. Biophys. Res. Commun. 93 (1980) 1114–1120.
[6] P.J. Barter, K.A. Rye, Cholesteryl ester transfer protein inhibition as a strategy to reduce cardiovascular risk, J. Lipid Res. 53 (2012) 1755–1766.
[7] R. Rao, J.J. Albers, G. Wolfbauer, H.J. Pownall, Molecular and macromolecular specificity of human plasma phospholipid transfer protein, Biochemistry 36 (1997) 3645–3653.
[8] P.J. Barter, J.J. Kastelein, Targeting cholesteryl ester transfer protein for the prevention and management of cardiovascular disease, J. Am. Coll. Cardiol. 47 (2006) 492–499.
[9] A. Kontush, M.J. Chapman, Functionally defective high-density lipoprotein: a new therapeutic target at the crossroads of dyslipidemia, inflammation, and atherosclerosis, Pharmacol. Rev. 58 (2006) 342–374.
[10] J.P. Segrest, S.C. Harvey, V. Zannis, Detailed molecular model of apolipoproteins A-I on the surface of high-density lipoproteins and its functional implications, Trends Cardiovasc. Med. 10 (2000) 246–252.
[11] H. Karlsson, P. Leanderson, C. Tagesson, M. Lindahl, Lipoproteomics II: mapping of proteins in high-density lipoprotein using two-dimensional gel electrophoresis and mass spectrometry, Proteomics 5 (2005) 1431–1445.
[12] R.S. Rosenson, H.B. Brewer Jr., B. Ansell, et al., Translation of high-density lipoprotein function into clinical practice: current prospects and future challenges, Circulation 128 (2013) 1256–1267.
[13] R.S. Rosenson, H.B. Brewer, M.J. Chapman, et al., HDL measures, particles heterogeneity, proposed nomenclature and relation to atherosclerotic cardiovascular events, Clin. Chem. 57 (2011) 392–410.
[14] J.M. Rash, G.H. Rothblat, C.E. Sparks, Lipoprotein apoliprotein synthesis by human hepatoma cells in culture, Biochim. Biophys. Acta 666 (1981) 294–298.
[15] M.J. Chapman, W. Le Goff, M. Guerin, A. Kontush, Cholesteryl ester transfer protein: at the heart of the action of lipid-modulating therapy with statins, fibrates, niacin, and cholesteryl ester transfer protein inhibitors, Eur. Heart J. 31 (2010) 149–164.
[16] J. Maric, R.S. Kiss, V. Franklin, Y.L. Marcel, Intracellular lipidation of newly synthesized apolipoprotein A-I in primary murine hepatocytes, J. Biol. Chem. 280 (2005) 39942–39949.

[17] C.L. Wellington, E.K. Walker, A. Suares, et al., ABCA1 mRNA and protein distribution patterns predict multiple different roles and levels of regulation, Lab. Invest. 82 (2002) 273–283.
[18] G.R. Castro, C.J. Fielding, Early incorporation of cell-derived cholesterol into pre-beta-migrating high-density lipoprotein, Biochemistry 27 (1988) 25–29.
[19] A.R. Tall, D. Sammett, G.M. Vita, R. Deckelbaum, T. Olivecrona, Lipoprotein lipase enhances the cholesteryl ester transfer protein-mediated transfer of cholesteryl ester from high density lipoproteins to very low density lipoproteins, J. Biol. Chem. 259 (1984) 9587–9594.
[20] J. Savel, M. Lafitte, Y. Pucheu, et al., Molecular cloning low levels of HDL-cholesterol and atherosclerosis, a variable relationship—a review of LCAT deficiency, Vasc. Health Risk Manag. 8 (2012) 357–361.
[21] X. Rousset, B. Vaisman, M. Amar, A.A. Sethi, A.T. Remaley, Lecithin:cholesterol acyltransferase—from biochemistry to role in cardiovascular disease, Curr. Opin. Endocrinol. Diabetes Obes. 16 (2009) 163–171.
[22] V. Bowry, K.K. Stanley, R. Stocker, High density lipoprotein is the major carrier of lipid hydroperoxides in human blood plasma from fasting donors, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 10316–10320.
[23] F. Robbesyn, V. Garcia, N. Auge, et al., HDL counterbalance the proinflammatory effect of oxidized LDL by inhibiting intracellular reactive oxygen species rise, proteasome activation, and subsequent NF-KB activation in smooth muscle cells, FASEB J. 17 (2003) 743–745.
[24] C.M. Lee, C.T. Chien, P.Y. Chang, et al., High-density lipoprotein antagonizes oxidized low-density lipoprotein by suppressing oxygen free-radical formation and preserving nitric oxide bioactivity, Atherosclerosis 183 (2005) 251–258.
[25] S.J. Nicholls, G.J. Dusting, B. Cutri, et al., Reconstituted HDL inhibits the acute pro-oxidant and proinflammatory vascular changes induced by a periarterial collar in normocholesterolemic rabbits, Circulation 111 (2005) 1543–1550.
[26] M. Navab, S.Y. Hama, C.J. Cooke, et al., Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: step 1, J. Lipid Res. 41 (2000) 1481–1494.
[27] M. Navab, S.Y. Hama, G. Anantharamaiah, et al., Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: steps 2 and 3, J. Lipid Res. 41 (2000) 1495–1508.
[28] K. Greenow, N.J. Pearce, D.P. Ramji, The key role of apolipoprotein E in atherosclerosis, J. Mol. Med. 83 (2005) 329–342.
[29] S.T. Kunitake, M.R. Jarvis, R.L. Hamilton, J.P. Kane, Binding of transition metals by apolipoprotein A-I-containing plasma lipoproteins: inhibition of oxidation of low density lipoproteins, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 6993–6997.
[30] H.W. Eckerson, C.M. Wyte, B.N. La Du, The human serum paraoxonase/arylesterase polymorphism, Am. J. Hum. Genet. 35 (1993) 1126–1138.
[31] M. Aviram, M. Rosenblat, C.L. Bisgaier, R.S. Newton, S.L. Primo-Parmo, B.N. La Du, Paraoxonase inhibits high-density lipoprotein oxidation and preserves its functions: a possible peroxidative role for paraoxonase, J. Clin. Invest. 101 (1998) 1581–1590.
[32] B. Mackness, G.K. Davies, W. Turkie, et al., Paraoxonase status in coronary heart disease: are activity and concentration more important than genotype? Arterioscler. Thromb. Vasc. Biol. 21 (2001) 1451–1457.
[33] R.W. James, S.P. Deakin, The importance of high-density lipoproteins for paraoxonase-1 secretion, stability, and activity, Free Radic. Biol. Med. 37 (2004) 1986–1994.

[34] B. Mackness, P. Durrington, P. McElduff, et al., Low paraoxonase activity predicts coronary events in the Caerphilly Prospective Study, Circulation 107 (2003) 2775–2779.
[35] D.A. Six, E.A. Dennis, The expanding superfamily of phospholipase A(2) enzymes: classification and characterization, Biochim. Biophys. Acta 1488 (2000) 1–19.
[36] K. Karasawa, Clinical aspects of plasma platelet activating factor-acetylhydrolase, Biochim. Biophys. Acta 1761 (2006) 1359–1372.
[37] A.D. Watson, M. Navab, S.Y. Hama, et al., Effect of platelet activating factor-acetylhydrolase on the formation and action of minimally oxidized low density lipoprotein, J. Clin. Invest. 95 (1995) 774–782.
[38] A.M. Evangelou, Platelet-activating factor (PAF): implications for coronary heart and vascular diseases, Prostaglandins Leukot. Essent. Fatty Acids 50 (1994) 1–28.
[39] G.W. Cockerill, K.A. Rye, J.R. Gamble, M.A. Vadas, P.J. Barter, High-density lipoproteins inhibit cytokine-induced expression of endothelial cell adhesion molecules, Arterioscler. Thromb. Vasc. Biol. 15 (1995) 1987–1994.
[40] L. Calabresi, G. Franceschini, C.R. Sirtori, et al., Inhibition of VCAM-1 expression in endothelial cells by reconstituted high density lipoproteins, Biochem. Biophys. Res. Commun. 238 (1997) 61–65.
[41] P.W. Baker, K.A. Rye, J.R. Gamble, M.A. Vadas, P.J. Barter, Ability of reconstituted high density lipoproteins to inhibit cytokine-induced expression of vascular cell adhesion molecule-1 in human umbilical vein endothelial cells, J. Lipid Res. 40 (1999) 345–353.
[42] P. Xia, M.A. Vadas, K.A. Rye, P.J. Barter, J.R. Gamble, High density lipoproteins (HDL) interrupt the sphingosine kinase signaling pathway: a possible mechanism for protection against atherosclerosis by HDL, J. Biol. Chem. 274 (1999) 33143–33147.
[43] G.D. Norata, E. Callegari, M. Marchesi, G. Chiesa, P. Eriksson, A.L. Catapano, High-density lipoproteins induce transforming growth factor-beta 2 expression in endothelial cells, Circulation 111 (2005) 2805–2811.
[44] M. Gomaraschi, N. Basilico, F. Sisto, et al., High-density lipoproteins attenuate interleukin-6 production in endothelial cells exposed to pro-inflammatory stimuli, Biochim. Biophys. Acta 1736 (2005) 136–143.
[45] B.J. Van Lenten, M. Navab, D. Shih, A.M. Fogelman, A.J. Lusis, The role of high-density lipoproteins in oxidation and inflammation, Trends Cardiovasc. Med. 11 (2001) 155–161.
[46] M. Navab, G. Ananthramaiah, S.T. Reddy, et al., The oxidation hypothesis of atherogenesis: the role of oxidized phospholipids and HDL, J. Lipid Res. 45 (2004) 993–1007.
[47] A. Furnkranz, A. Schober, V.N. Bochkov, et al., Oxidized phospholipids trigger atherogenic inflammation in murine arteries, Arterioscler. Thromb. Vasc. Biol. 25 (2005) 633–638.
[48] G. Theilmeier, B. de Geest, P.P. van Veldhoven, et al., HDL-associated PAF-AH reduces endothelial adhesiveness in apoE −/− mice, FASEB J. 14 (2000) 2032–2039.
[49] J.H. Griffin, K. Kojima, C.L. Banka, L.K. Curtiss, J.A. Fernandez, High-density lipoprotein enhancement of anticoagulant activities of plasma protein S and activated protein C, J. Clin. Invest. 103 (1999) 219–227.
[50] C. Mineo, H. Deguchi, J.H. Griffin, P.W. Shaul, Endothelial and antithrombotic actions of HDL, Circ. Res. 98 (2006) 1352–1364.
[51] I. Suc, I. Escargueil-Blanc, M. Troly, R. Salvayre, A. Negre-Salvayre, HDL and Apo A prevent cell death of endothelial cells induced by oxidized LDL, Arterioscler. Thromb. Vasc. Biol. 17 (1997) 2158–2166.

[52] M. Sugano, K. Tsuchida, N. Makino, High-density lipoproteins protect endothelial cells from tumor necrosis factor-alpha-induced apoptosis, Biochem. Biophys. Res. Commun. 272 (2000) 872–876.
[53] Y.G. Kwon, J.K. Min, K.M. Kim, D.J. Lee, T.R. Billiar, Y.M. Kim, Sphingosine 1-phosphate protects human umbilical vein endothelial cells from serum-deprived apoptosis by nitric oxide production, J. Biol. Chem. 276 (2001) 10627–10633.
[54] J.R. Nofer, M. van der Giet, M. Tölle, et al., HDL induces NO dependent vasorelaxation via the lysophospholipid receptor S1P3, J. Clin. Invest. 113 (2004) 569–581.
[55] B. Zhang, H. Tomura, A. Kuwabara, et al., Correlation of high density lipoprotein (HDL)-associated sphingosine 1-phosphate with serum levels of HDL-cholesterol and apolipoproteins, Atherosclerosis 178 (2005) 199–205.
[56] B.G. Drew, N.H. Fidge, G. Gallon-Beaumier, B.E. Kemp, B.A. Kingwell, High-density lipoprotein and apolipoprotein AI increase endothelial NO synthase activity by protein association and multisite phosphorylation, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 6999–7004.
[57] I.S. Yuhanna, Y. Zhu, B.E. Cox, et al., High-density lipoprotein binding to scavenger receptor-BI activates endothelial nitric oxide synthase, Nat. Med. 7 (2001) 853–857.
[58] G.D. Norata, E. Callegari, H. Inoue, A.L. Catapano, HDL3 induces cyclooxygenase-2 expression and prostacyclin release in human endothelial cells via a p38 MAPK/CRE-dependent pathway: effects on COX-2/PGI-synthase coupling, Arterioscler. Thromb. Vasc. Biol. 24 (2004) 871–877.
[59] T. Kimura, K. Sato, E. Malchinkhuu, et al., High-density lipoprotein stimulates endothelial cell migration and survival through sphingosine 1-phosphate and its receptors, Arterioscler. Thromb. Vasc. Biol. 23 (2003) 1283–1288.
[60] K. Tamama, H. Tomura, K. Sato, et al., High-density lipoprotein inhibits migration of vascular smooth muscle cells through its sphingosine 1-phosphate component, Atherosclerosis 178 (2005) 19–23.
[61] H. Deguchi, J.A. Fernandez, T.M. Hackeng, C.L. Banka, J.H. Griffin, Cardiolipin is a normal component of human plasma lipoproteins, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 1743–1748.
[62] L.Y. Chen, J.L. Mehta, Inhibitory effect of high-density lipoprotein on platelet function is mediated by increase in nitric oxide synthase activity in platelets, Life Sci. 55 (1994) 1815–1821.
[63] J. Ma, X.L. Liao, B. Lou, M.P. Wu, Role of apolipoprotein A-I in protecting against endotoxin toxicity, Acta Biochim. Biophys. Sin. 36 (2004) 419–424.
[64] D. Pajkrt, J.E. Doran, F. Koster, et al., Antiinflammatory effects of reconstituted high-density lipoprotein during human endotoxemia, J. Exp. Med. 184 (1996) 1601–1608.
[65] L.L. Stoll, G.M. Denning, N.L. Weintraub, Potential role of endotoxin as a proinflammatory mediator of atherosclerosis, Arterioscler. Thromb. Vasc. Biol. 24 (2004) 2227–2236.
[66] R. Yu, B. Yekta, L. Vakili, et al., Proatherogenic high-density lipoprotein, vascular inflammation, and mimetic peptides, Curr. Atheroscler. Rep. 10 (2008) 171–176.
[67] P.K. Shah, K.Y. Chyu, Apolipoprotein A-I mimetic peptides: potential role in atherosclerosis management, Trends Cardiovasc. Med. 15 (2005) 291–296.
[68] M. Navab, G.M. Anantharamaiah, S.T. Reddy, B.J. Van Lenten, B.J. Ansell, A.M. Fogelman, Mechanisms of disease: proatherogenic HDL—an evolving field, Nat. Clin. Pract. Endocrinol. Metab. 2 (2006) 504–511.
[69] M. Tölle, T. Huang, M. Schuchardt, et al., High-density lipoprotein loses its anti-inflammatory capacity by accumulation of pro-inflammatory-serum amyloid A, Cardiovasc. Res. 94 (2012) 154–162.

[70] R.P. Dullaart, J.F. de Boer, W. Annema, U.J. Tietge, The inverse relation of HDL anti-oxidative functionality with serum amyloid A is lost in metabolic syndrome, Obesity 21 (2013) 361–366.
[71] D. Drayna, A.S. Jarnagin, J. McLean, Cloning and sequencing of human cholesteryl ester transfer protein cDNA, Nature 327 (1987) 632–634.
[72] A.R. Tall, Plasma lipid transfer proteins, Annu. Rev. Biochem. 64 (1995) 235–257.
[73] P.J. Barter, CETP and atherosclerosis, Arterioscler. Thromb. Vasc. Biol. 20 (2000) 2029–2031.
[74] P.J. Barter, C.J. Hopkins, G.D. Calver, Transfers and exchanges of esterified cholesterol between plasma lipoproteins, Biochem. J. 208 (1982) 1–7.
[75] Y.L. Marcel, M. McPherson, H. Hogue, et al., Distribution and concentration of cholesteryl ester transfer protein in plasma of normolipemic subjects, J. Clin. Invest. 85 (1990) 10–17.
[76] T. Gautier, D. Masson, J.P. de Barros, et al., Human apolipoprotein CI accounts for the ability of plasma high density lipoproteins to inhibit the cholesteryl ester transfer protein activity, J. Biol. Chem. 275 (2000) 37504–37509.
[77] X. Wang, D.M. Driscoll, R.E. Morton, Molecular cloning and expression of lipid transfer inhibitor protein reveals its identity with apolipoprotein F, J. Biol. Chem. 274 (1999) 1814–1820.
[78] K.J. Williams, I. Tabas, The response-to-retention hypothesis of early atherogenesis, Arterioscler. Thromb. Vasc. Biol. 15 (1995) 551–561.
[79] B.H. Chung, J.P. Segrest, F. Franklin, In vitro production of beta-very low density lipoproteins and small, dense low density lipoproteins in mildly hypertriglyceridemic plasma: role of activities of lecithin:cholesterol acyl transferase, cholesteryl ester transfer protein and lipoprotein lipase, Atherosclerosis 141 (1998) 209–225.
[80] H.H. Newnham, P.J. Barter, Synergistic effects of lipid transfers and hepatic lipase in the formation of very small high density lipoproteins during incubation of human plasma, Biochim. Biophys. Acta 1044 (1990) 57–64.
[81] H.Q. Liang, K.A. Rye, P.J. Barter, Dissociation of lipid-free apolipoprotein A-I from high density lipoproteins, J. Lipid Res. 35 (1994) 1187–1199.
[82] K.A. Rye, N.J. Hime, P.J. Barter, Evidence that CETP-mediated reductions in reconstituted high density lipoprotein size involve particle fusion, J. Biol. Chem. 272 (1997) 5953–5960.
[83] S. Lund-Katz, P.M. Laplaud, M.C. Phillips, M.J. Chapman, Apolipoprotein B-100 conformation and particle surface charge in human LDL subspecies: implication for LDL receptor interaction, Biochemistry 37 (1998) 12867–12874.
[84] C.J. Mann, F.T. Yen, A.M. Grant, B.E. Bihain, Mechanism of cholesteryl ester transfer in hypertriglyceridemia, J. Clin. Invest. 88 (1991) 2059–2066.
[85] X.Y. Qiu, A. Mistry, M.J. Ammirati, et al., Crystal structure of cholesteryl ester transfer protein reveals a long tunnel and four bound lipid molecules, Nat. Struct. Mol. Biol. 14 (2007) 106–113.
[86] P.J. Barter, M.E. Jones, Kinetic studies of the transfer of esterified cholesterol between human-plasma low and high-density lipoproteins, J. Lipid Res. 21 (1980) 238–249.
[87] J. Ihm, D.M. Quinn, S.J. Busch, B. Chataing, J.A. Harmony, Kinetics of plasma protein-catalyzed exchange of phosphatidylcholine and cholesteryl ester between plasma lipoproteins, J. Lipid Res. 23 (1982) 1328–1341.
[88] L. Zhang, F. Yan, S. Zhang, et al., Structural basis of transfer between lipoproteins by cholesteryl ester transfer protein, Nat. Chem. Biol. 8 (2012) 342–349.
[89] L. Gomez Rosso, M.B. Benitez, M.C. Fornari, V. Berardi, S. Lynch, L. Schreier, et al., Alterations in cell adhesion molecules and other biomarkers of cardiovascular disease in patients with metabolic syndrome, Atherosclerosis 199 (2008) 415–423.

[90] R.I. Coniglio, T. Meroño, H. Montiel, et al., HOMA-IR and non-HDL-C as a predictors of high cholesterol ester transfer protein activity in patients at risk for type 2 diabetes, Clin. Biochem. 45 (2012) 566–570.
[91] J.T. Jonker, Y. Wang, W. de Haan, et al., Pioglitazone decreases plasma cholesteryl ester transfer protein mass, associated with a decrease in hepatic triglyceride content, in patients with type 2 diabetes, Diabetes Care 33 (2010) 1625–1628.
[92] H.C. Oliveira, E.C. de Faria, Cholesteryl ester transfer protein: the controversial relation to atherosclerosis and emerging new biological roles, IUBMB Life 63 (2011) 248–257.
[93] R.W. Clark, R.B. Ruggeri, D. Cunningham, M.J. Bamberger, Description of the torcetrapib series of cholesterol ester transfer protein inhibitors, including the mechanism of action, J. Lipid Res. 47 (2006) 537–552.
[94] P.J. Barter, M. Caulfield, M. Eriksson, et al., Effects of torcetrapib in patients at high risk for coronary events, N. Engl. J. Med. 357 (2007) 2109–2122.
[95] J.J. Kastelein, S.I. van Leuven, L. Burgess, et al., Effect of torcetrapib on carotid atherosclerosis in familial hypercholesterolemia, N. Engl. J. Med. 356 (2007) 1620–1630.
[96] M.L. Bots, F.L. Visseren, G.W. Evans, et al., Torcetrapib and carotid intima-media thickness in mixed dyslipidaemia (RADIANCE 2 study): a randomised, double-blind trial, Lancet 370 (2007) 153–160.
[97] P.J. Barter, Lessons learned from the Investigation of Lipid Level Management to Understand its Impact in Atherosclerotic Events (ILLUMINATE) trial, Am. J. Cardiol. 104 (2009) 10E–15E.
[98] E.S. Stroes, J.J. Kastelein, A. Bénardeau, et al., Dalcetrapib: no off-target toxicity on blood pressure or on genes related to the renin-angiotensin-aldosterone system in rats, Br. J. Pharmacol. 158 (2009) 1763–1770.
[99] M.J. Forrest, D. Bloomfield, R.J. Briscoe, et al., Torcetrapib-induced blood pressure elevation is independent of CETP inhibition and is accompanied by increased circulating levels of aldosterone, Br. J. Pharmacol. 154 (2008) 1465–1473.
[100] M. Guerin, W. Le Goff, E. Duchene, et al., Inhibition of CETP by torcetrapib attenuates the atherogenicity of postprandial TG-rich lipoproteins in type IIB hyperlipidemia, Arterioscler. Thromb. Vasc. Biol. 28 (2008) 148–154.
[101] H. Okamoto, F. Yonemori, K. Wakitani, T. Minowa, K. Maeda, H. Shinkai, A cholesteryl ester transfer protein inhibitor attenuates atherosclerosis in rabbits, Nature 406 (2000) 203–207.
[102] E.J. Niesor, E. von der Marck, M. Brousse, C. Maugeais, Inhibition of cholesteryl ester transfer protein (CETP): different in vitro characteristics of RO4607381/JTT-705 and torcetrapib (TOR), Atherosclerosis 199 (2008) 231.
[103] G.J. de Grooth, J.A. Kuivenhoven, A.F. Stalenhoef, et al., Efficacy and safety of a novel cholesteryl ester transfer protein inhibitor, JTT-705, in humans: a randomized phase II dose–response study, Circulation 105 (2002) 2159–2165.
[104] J.A. Kuivenhoven, G.J. de Grooth, H. Kawamura, et al., Effectiveness of inhibition of cholesteryl ester transfer protein by JTT-705 in combination with pravastatin in type II dyslipidemia, Am. J. Cardiol. 95 (2005) 1085–1088.
[105] E.A. Stein, E.M. Roth, J.M. Rhyne, T. Burgess, D. Kallend, J.G. Robinson, Safety and tolerability of dalcetrapib (RO4607381/JTT-705): results from a 48- week trial, Eur. Heart J. 31 (2010) 480–488.
[106] M. Ranalletta, K.K. Bierilo, Y. Chen, et al., Biochemical characterization of cholesteryl ester transfer protein inhibitors, J. Lipid Res. 51 (2010) 2739–2752.
[107] R. Krishna, M.S. Anderson, A.J. Bergman, et al., Effect of the cholesteryl ester transfer protein inhibitor, anacetrapib, on lipoproteins in patients with dyslipidaemia and on 24-h ambulatory blood pressure in healthy individuals: two double-blind, randomised placebo-controlled phase I studies, Lancet 370 (2007) 1907–1914.

[108] D. Bloomfield, G.L. Carlson, A. Sapre, et al., Efficacy and safety of the cholesteryl ester transfer protein inhibitor anacetrapib as monotherapy and coadministered with atorvastatin in dyslipidemic patients, Am. Heart J. 157 (2009) 352–360.
[109] C.P. Cannon, H.M. Dansky, M. Davidson, et al., Design of the DEFINE trial: determining the EFficacy and tolerability of CETP INhibition with AnacEtrapib, Am. Heart J. 158 (2009) 513–519.
[110] C.P. Cannon, S. Shah, H.M. Dansky, et al., Determining the efficacy and tolerability investigators, safety of anacetrapib in patients with or at high risk for coronary heart disease, N. Engl. J. Med. 363 (2010) 2406–2415.
[111] L. Yvan-Charvet, J. Kling, T. Pagler, et al., Cholesterol efflux potential and antiinflammatory properties of high-density lipoprotein after treatment with niacin or anacetrapib, Arterioscler. Thromb. Vasc. Biol. 30 (2010) 1430–1438.
[112] D.E. Gutstein, R. Krishna, D. Johns, et al., Anacetrapib, a novel CETP inhibitor: pursuing a new approach to cardiovascular risk reduction, Clin. Pharmacol. Ther. 91 (2012) 109–122.
[113] H. Shinkai, Cholesteryl ester transfer-protein modulator and inhibitors and their potential for the treatment of cardiovascular diseases, Vasc. Health Risk Manag. 8 (2012) 323–331.
[114] G. Cao, T.P. Beyer, Y. Zhang, et al., Evacetrapib is a novel, potent, and selective inhibitor of cholesteryl ester transfer protein that elevates HDL cholesterol without inducing aldosterone or increasing blood pressure, J. Lipid Res. 52 (2011) 2169–2176.
[115] S.J. Nicholls, H.B. Brewer, J.J. Kastelein, et al., Effects of the CETP inhibitor evacetrapib administered as monotherapy or in combination with statin on HDL and LDL cholesterol. A randomized controlled trial, JAMA 306 (2011) 2099–2109.
[116] J.R. Day, J.J. Albers, C.E. Lofton-Day, et al., Complete cDNA encoding human phospholipid transfer protein from human endothelial cells, J. Biol. Chem. 269 (1994) 9388–9391.
[117] E. Hailman, J.J. Albers, G. Wolfbauer, A.Y. Tu, S.D. Wright, Neutralization and transfer of lipopolysaccharide by phospholipid transfer protein, J. Biol. Chem. 271 (1996) 12172–12178.
[118] J.J. Albers, J.R. Day, G. Wolfbauer, H. Kennedy, S. Vuletic, M.C. Cheung, Impact of site-specific N-glycosylation on cellular secretion, activity and specific activity of the plasma phospholipid transfer protein, Biochim. Biophys. Acta 1814 (2011) 908–911.
[119] T. Oka, T. Kujiraoka, M. Ito, Distribution of phospholipid transfer protein in human plasma: presence of two forms of phospholipid transfer protein, one catalytically active and the other inactive, J. Lipid Res. 41 (2000) 1651–1657.
[120] M. Kärkkäinen, T. Oka, V.M. Olkkonen, et al., Isolation and partial characterization of the inactive and active forms of human plasma phospholipid transfer protein (PLTP), J. Biol. Chem. 277 (2002) 15413–15418.
[121] M.C. Cheung, G. Wolfbauer, J.J. Albers, Different phospholipid transfer protein complexes contribute to the variation in plasma PLTP specific activity, Biochim. Biophys. Acta 1811 (2011) 343–347.
[122] M.T. Janis, J. Metso, H. Lankinen, et al., Apolipoprotein E activates the low-activity form of human phospholipid transfer protein, Biochem. Biophys. Res. Commun. 331 (2005) 333–340.
[123] J.J. Albers, S. Vuletic, M.C. Cheung, Role of plasma phospholipid transfer protein in lipid and lipoprotein metabolism, Biochim. Biophys. Acta 1821 (2012) 345–357.
[124] A.R. Tall, S. Krumholz, T. Olivecrona, R.J. Deckelbaum, Plasma phospholipid transfer protein enhances transfer and exchange of phospholipids between very low density lipoproteins and high density lipoproteins during lipolysis, J. Lipid Res. 26 (1985) 842–851.

[125] X.C. Jiang, C. Bruce, J. Mar, et al., Targeted mutation of plasma phospholipid transfer protein gene markedly reduces high-density lipoprotein levels, J. Clin. Invest. 103 (1999) 907–914.
[126] X.C. Jiang, A.R. Tall, S. Qin, et al., Phospholipid transfer protein deficiency protects circulating lipoproteins from oxidation due to the enhanced accumulation of vitamin E, J. Biol. Chem. 277 (2002) 31850–31856.
[127] B. Bailey, I. Ruel, A. Mafinae, et al., Analysis of lipid transfer activity between model nascent HDL particles and plasma lipoproteins: implications for current concepts of nascent HDL maturation and genesis, J. Lipid Res. 51 (2010) 785–797.
[128] G. Wolfbauer, J.J. Albers, J.F. Oram, Phospholipid transfer protein enhances removal of cellular cholesterol and phospholipids by high-density lipoprotein apolipoproteins, Biochim. Biophys. Acta 1439 (1999) 65–76.
[129] R. Vikstedt, J. Metso, J. Hakala, V.M. Olkkonen, C. Ehnholm, M. Jauhiainen, Cholesterol efflux from macrophage foam cell is enhanced by active phospholipid transfer protein through generation of two types of acceptor particles, Biochemistry 46 (2007) 11979–11986.
[130] J.F. Oram, G. Wolfbauer, C. Tang, W.S. Davidson, J.J. Albers, An amphipathic helical region of the N-terminal barrel of phospholipid transfer protein is critical for ABCA1-dependent cholesterol efflux, J. Biol. Chem. 283 (2008) 11541–11549.
[131] M.C. Cheung, G. Wolfbauer, H. Deguchi, J.A. Fernandez, J.H. Griffin, J.J. Albers, Human plasma phospholipid transfer protein specific activity is correlated with HDL size: implications for lipoprotein physiology, Biochim. Biophys. Acta 1791 (2009) 206–211.
[132] R.P. Dullaart, R. de Vries, L. Scheek, et al., Type 2 diabetes mellitus is associated with differential effects on plasma cholesteryl ester transfer protein and phospholipid transfer protein activities and concentrations, Scand. J. Clin. Lab. Invest. 64 (2004) 205–215.
[133] S.J. Murdoch, M.C. Carr, J.E. Hokanson, J.D. Brunzell, J.J. Albers, PLTP activity in premenopausal women. Relationship with lipoprotein lipase, HDL, LDL, body fat, and insulin resistance, J. Lipid Res. 41 (2000) 237–244.
[134] M.C. Cheung, R.H. Knopp, B. Retzlaff, H. Kennedy, G. Wolfbauer, J.J. Albers, Association of plasma phospholipid transfer protein activity with IDL and buoyant LDL: impact of gender and adiposity, Biochim. Biophys. Acta 1587 (2002) 53–59.
[135] M.H. Colhoun, M.R. Taskinen, J.D. Otvos, P. van den Berg, J. O'Connor, A. van Tol, Relationship of phospholipid transfer protein activity to HDL and apolipoprotein B-containing lipoproteins in subjects with and without type 1 diabetes, Diabetes 51 (2002) 3300–3305.
[136] S.C. Riemens, A. van Tol, W.J. Sluiter, R.P. Dullaart, Plasma phospholipid transfer protein activity is related to insulin resistance: impaired acute lowering by insulin in obese type II diabetic patients, Diabetologia 41 (1998) 929–934.
[137] S. Kaser, A. Sandhofer, B. Föger, et al., Influence of obesity and insulin sensitivity on phospholipid transfer protein activity, Diabetologia 44 (2001) 1111–1117.
[138] A. Schlitt, C. Bickel, P. Thumma, et al., High plasma phospholipid transfer protein levels as a risk factor for coronary artery disease, Arterioscler. Thromb. Vasc. Biol. 23 (2003) 1857–1862.
[139] H. Yatsuya, K. Tamakoshi, H. Hattori, et al., Serum phospholipid transfer protein mass as a possible protective factor for coronary heart diseases, Circ. J. 68 (2004) 11–16.
[140] E. Cavusoglu, J.D. Marmur, S. Chhabra, V. Chopra, C. Eng, X.C. Jiang, Relation of baseline plasma phospholipid transfer protein (PLTP) activity to left ventricular systolic dysfunction in patients referred for coronary angiography, Atherosclerosis 207 (2009) 261–265.
[141] X. Chen, A. Sun, A. Mansoor, et al., Plasma PLTP activity is inversely associated with HDL-C levels, Nutr. Metab. 6 (2009) 49–54.

[142] W. Schgoer, T. Mueller, M. Jauhiainen, et al., Low phospholipid transfer protein (PLTP) is a risk factor for peripheral atherosclerosis, Atherosclerosis 196 (2008) 219–226.
[143] T. Oka, S. Yamashita, T. Kujiraoka, et al., Distribution of human plasma PLTP mass and activity in hypo- and hyperalphalipoproteinemia, J. Lipid Res. 43 (2002) 1236–1243.
[144] G.M. Kostner, K. Oetti, M. Jauhiainen, C. Ehnholm, H. Esterbauer, H. Dieplinger, Human plasma phospholipid transfer protein accelerates exchange/transfer of alpha-tocopherol between lipoproteins and cells, Biochem. J. 305 (1995) 659–667.
[145] C. Desrumaux, V. Deckert, A. Athias, et al., Plasma phospholipid transfer protein prevents vascular endothelium dysfunction by delivering alpha-tocopherol to endothelial cells, FASEB J. 13 (1999) 883–892.
[146] D. Yan, M. Navab, C. Bruce, A.M. Fogelman, X.C. Jiang, PLTP deficiency improves the anti-inflammatory properties of HDL and reduces the ability of LDL to induce monocyte chemotactic activity, J. Lipid Res. 45 (2004) 1852–1858.
[147] X.P. Yang, D. Yan, C. Qiao, et al., Increased atherosclerotic lesions in apo E mice with plasma phospholipid transfer protein overexpression, Arterioscler. Thromb. Vasc. Biol. 23 (2003) 1601–1607.
[148] A. Klein, V. Deckert, M. Schneider, et al., Alpha-tocopherol modulates phosphatidylserine externalization in erythrocytes: relevance in phospholipid transfer protein-deficient mice, Arterioscler. Thromb. Vasc. Biol. 26 (2006) 2160–2167.
[149] C. Oslakovic, M.J. Krisinger, A. Andersson, M. Jauhiainen, C. Ehnholm, B. Dahlbäck, Anionic phospholipids lose their procoagulant properties when incorporated into high density lipoproteins, J. Biol. Chem. 284 (2009) 5896–5904.
[150] C. Oslakovic, M. Jauhiainen, C. Ehnholm, B. Dahlbäck, The role of phospholipid transfer protein in lipoprotein-mediated neutralization of the procoagulant effect of anionic liposomes, J. Thromb. Haemost. 8 (2010) 766–772.
[151] R. de Vries, G.M. Dallinga-Thie, A.J. Smit, B.H. Wolffenbuttel, A. van Tol, R.P. Dullaart, Elevated plasma phospholipid transfer protein activity is a determinant of carotid intima-media thickness in type 2 diabetes mellitus, Diabetologia 49 (2006) 398–404.
[152] E.C. Quintão, P.M. Cazita, Lipid transfer proteins: past, present and perspectives, Atherosclerosis 209 (2010) 1–9.
[153] A. Thompson, E. Di Angelantonio, N. Sarwar, et al., Association of cholesteryl ester transfer protein genotypes with CETP mass and activity, lipid levels, and coronary risk, JAMA 299 (2008) 2777–2788.
[154] S.E. Nissen, J.C. Tardif, S.J. Nicholls, et al., Effect of torcetrapib on the progression of coronary atherosclerosis, N. Engl. J. Med. 356 (2007) 1304–1316.
[155] A. Ritsch, H. Scharnagl, P. Eller, et al., Cholesteryl ester transfer protein and mortality in patients undergoing coronary angiography: the Ludwigshafen Risk and Cardiovascular Health Study, Circulation 121 (2010) 366–374.
[156] A.V. Khera, M.L. Wolfe, C.P. Cannon, J. Qin, D.J. Rader, On-statin cholesteryl ester transfer protein mass and risk of recurrent coronary events (from the pravastatin or atorvastatin evaluation and infection therapy–thrombolysis in myocardial infarction 22 [PROVE-IT-TIMI 22] Study), Am. J. Cardiol. 106 (2010) 451–456.
[157] M. Vergeer, M. Boekholdt, M.S. Sandhu, et al., Genetic variation at the phospholipid transfer protein locus affects its activity and high-density lipoprotein size and is a novel marker of cardiovascular disease susceptibility, Circulation 122 (2010) 470–477.
[158] S.J. Robins, A. Lyass, R.W. Brocia, J.M. Massaro, R.S. Vasan, Plasma lipid transfer proteins and cardiovascular disease. The Framingham Heart Study, Atherosclerosis 228 (2013) 230–236.

[159] G. Franceschini, C.R. Sirtori, A. Capurso, K.H. Weisgraber, R.W. Mahley, A-IMilano poprotein. Decreased high density lipoprotein cholesterol levels with significant lipoprotein modifications and without clinical atherosclerosis in an italian family, J. Clin. Invest. 66 (1980) 892–900.
[160] G. Franceschini, C.R. Sirtori, E. Bosisio, et al., Relationship of the phenotypic expression of the A-IMilano apoprotein with plasma lipid and lipoprotein patterns, Atherosclerosis 58 (1985) 159–174.
[161] V. Gualandri, G.B. Orsini, A. Cerrone, G. Franceschini, C.R. Sirtori, Familial associations of lipids and lipoproteins in a highly consanguineous population: the Limone Sul Garda Study, Metabolism 34 (1985) 212–221.
[162] C.R. Sirtori, L. Calabresi, G. Franceschini, et al., Cardiovascular status of carriers of the apolipoprotein A-I(Milano) mutant: the Limone sul Garda study, Circulation 103 (2001) 1949–1954.
[163] G. Franceschini, L. Calabresi, G. Chiesa, et al., Increased cholesterol efflux potential of sera from Apo A-I Milano carriers and transgenic mice, Arterioscler. Thromb. Vasc. Biol. 19 (1999) 1257–1262.
[164] C. Parolini, G. Chiesa, Y. Zhu, et al., Targeted replacement of mouse apolipoprotein A-I with human ApoA-I or the mutant ApoAIMilano. Evidence of APOA-IM impaired hepatic secretion, J. Biol. Chem. 278 (2003) 4740–4746.
[165] C. Parolini, G. Chiesa, E. Gong, et al., Apolipoprotein A-I and the molecular variant apoA-I(Milano): evaluation of the antiatherogenic effects in knock-in mouse model, Atherosclerosis 183 (2005) 222–229.
[166] C. Lebherz, J. Sanmiguel, J.M. Wilson, D.J. Rader, Gene transfer of wild-type apoA-I and apoA-I Milano reduce atherosclerosis to a similar extent, Cardiovasc. Diabetol. 6 (2007) 6–15.
[167] A.V. Khera, M. Cuchel, M. de la Llera-Moya, et al., Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis, N. Engl. J. Med. 364 (2011) 127–135.
[168] A.C. Lo Prete, C.H. Dina, C.H. Azevedo, et al., In vitro simultaneous transfer of lipids to HDL in coronary artery disease and in statin treatment, Lipids 44 (2009) 917–924.
[169] C.H. Azevedo, M. Wajngarten, A.C. Lo Prete, J. Diament, R.C. Maranhão, Simultaneous transfer of cholesterol, triglycerides, and phospholipids to high-density lipoprotein in aging subjects with or without coronary artery disease, Clinics (Sao Paulo) 66 (2011) 1543–1548.
[170] A.H. Giribela, N.R. Melo, M.C. Latrilha, E.C. Baracat, R.C. Maranhão, HDL concentration, lipid transfer to HDL, and HDL size in normolipidemic nonobese menopausal women, Int. J. Gynaecol. Obstet. 104 (2009) 117–120.
[171] R.C. Maranhão, F.R. Freitas, C.M. Strunz, R.D. Santos, A.J. Mansur, A.P. Mansur, Lipid transfers to HDL are predictors of precocious clinical coronary heart disease, Clin. Chim. Acta 413 (2012) 502–505.
[172] M.P. Bertato, C.P. Oliveira, B.L. Wajchenberg, A.C. Lerario, R.C. Maranhão, Plasma kinetics of an LDL-like nanoemulsion and lipid transfer to HDL in subjects with glucose intolerance, Clinics (Sao Paulo) 67 (2012) 347–353.
[173] A. Casella-Filho, A.C. Chagas, R.C. Maranhão, et al., Effect of exercise training on plasma levels and functional properties of high-density lipoprotein cholesterol in the metabolic syndrome, Am. J. Cardiol. 107 (2011) 1168–1172.
[174] C.P. Oliveira, R.C. Maranhão, M.P. Bertato, B.L. Wajchenberg, A.C. Lerario, Removal from the plasma of the free and esterified forms of cholesterol and transfer of lipids to HDL in type 2 diabetes mellitus patients, Lipids Health Dis. 11 (2012) 65.
[175] A.C. Feitosa, G.S. Feitosa-Filho, F.R. Freitas, B.L. Wajchenberg, R.C. Maranhão, Lipoprotein metabolism in patients with type 1 diabetes under intensive insulin treatment, Lipids Health Dis. 12 (2013) 15.

[176] L.R. Martinez, R.D. Santos, M.H. Miname, D.F. Deus, E.S. Lima, R.C. Maranhão, Transfer of lipids to high-density lipoprotein (HDL) is altered in patients with familial hypercholesterolemia, Metabolism 62 (2013) 1061–1064.
[177] M.P. Rocha, R.C. Maranhão, T.M. Seydell, et al., Metabolism of triglyceride-rich lipoproteins and lipid transfer to high-density lipoprotein in young obese and normal-weight patients with polycystic ovary syndrome, Fertil. Steril. 93 (2010) 1948–1956.
[178] G.A. Sigal, G. Medeiros-Neto, J.C. Vinagre, J. Diament, R.C. Maranhão, Lipid metabolism in subclinical hypothyroidism: plasma kinetics of triglyceride-rich lipoproteins and lipid transfers to high-density lipoprotein before and after levothyroxine treatment, Thyroid 21 (2011) 347–353.
[179] C.G. Puk, E.A. Bocchi, A.C. Lo Prete, S.M. Ferreira, N.A. Stolf, R.C. Maranhão, Transfer of cholesterol and other lipids from a lipid nanoemulsion to high-density lipoprotein in heart transplant patients, J. Heart Lung Transplant. 28 (2009) 1075–1080.
[180] E.N. Daminelli, C. Spada, A. Treitinger, T.V. Oliveira, M.C. Latrilha, R.C. Maranhão, Alterations in lipidtransfer to high-density lipoprotein (HDL) and activity of paraoxonase-1 in HIV+ patients, Rev. Inst. Med. Trop. Sao Paulo 50 (2008) 223–227.
[181] J.C. Vinagre, C.G. Vinagre, F.S. Pozzi, E. Slywitch, R.C. Maranhão, Metabolism of triglyceride-rich lipoproteins and transfer of lipids to high-density lipoproteins (HDL) in vegan and omnivore subjects, Nutr. Metab. Cardiovasc. Dis. 23 (2013) 61–67.
[182] A.C. Gagliardi, R.C. Maranhão, H.P. de Sousa, E.J. Schaefer, R.D. Santos, Effects of margarines and butter consumption on lipid profiles, inflammation markers and lipid transfer to HDL particles in free-living subjects with the metabolic syndrome, Eur. J. Clin. Nutr. 64 (2010) 1141–1149.
[183] J.L. Silva, C.G. Vinagre, A.T. Morikawa, M.J. Alves, C.H. Mesquita, R.C. Maranhão, Resistance training changes LDL metabolism in normolipidemic subjects: a study with a nanoemulsion mimetic of LDL, Atherosclerosis 219 (2011) 532–537.
[184] M. Vaisberg, A.L. Bachi, M.C. Latrilha, G.S. Dioguardi, S.P. Bydlowski, R.C. Maranhão, Lipid transfer to HDL is higher in marathon runners than in sedentary subjects, but is acutely inhibited during the run, Lipids 47 (2012) 679–686.
[185] R.D. Santos, W. Hueb, A.A. Oliveira, J.A. Ramires, R.C. Maranhão, Plasma kinetics of a cholesterol-rich emulsion in subjects with or without coronary artery disease, J. Lipid Res. 44 (2003) 464–469.
[186] R.D. Couto, L.A. Dallan, L.A. Lisboa, C.H. Mesquita, C.G. Vinagre, R.C. Maranhão, Deposition of free cholesterol in the blood vessels of patients with coronary artery disease: a possible novel mechanism for atherogenesis, Lipids 42 (2007) 411–418.
[187] I. Sudano, L.E. Spieker, G. Noll, R. Corti, R. Weber, T.F. Lüscher, Cardiovascular disease in HIV infection, Am. Heart J. 151 (2006) 1147–1155.
[188] M. Guerin, W. Le Goff, T.S. Lassel, et al., Atherogenic role of elevated CE transfer from HDL to VLDL (1) and dense LDL in type 2 diabetes: impact of the degree of hypertriglyceridemia, Arterioscler. Thromb. Vasc. Biol. 21 (2001) 282–288.
[189] T. Hayek, N. Azrolan, R.B. Verdery, et al., Hypertriglyceridemia and cholesteryl ester transfer protein interact to dramatically alter high density lipoprotein levels, particle sizes, and metabolism. Studies in transgenic mice, J. Clin. Invest. 92 (1993) 1143–1152.
[190] A. Inazu, M.L. Brown, C.B. Hesler, et al., Increased high-density lipoprotein levels caused by a common cholesteryl-ester transfer protein gene mutation, N. Engl. J. Med. 323 (1990) 1234–1238.

CHAPTER TWO

Diagnosis of Infection in Critical Care

Belén Prieto[*], **Francisco V. Álvarez Menéndez**[*,†,1]
[*]Clinical Biochemistry, Laboratory of Medicine, Hospital Universitario Central de Asturias, Oviedo, Spain
[†]Biochemistry and Molecular Biology Department, University of Oviedo, Oviedo, Spain
[1]Corresponding author: e-mail address: falvarezmen@gmail.com

Contents

Abstract

Sepsis is the primary cause of death in the intensive care unit. The prevention of sepsis complications requires an early and accurate diagnosis as well as the appropriate monitoring. A deep knowledge of the immunologic basis of sepsis is essential to better understand the scope of incorporating a new marker into clinical practice.

Besides revising this theoretical aspect, the current available tools for bacterial identification have been briefly reviewed as well as a variety of new markers showing either well-recognized or potential usefulness for diagnosis and prognosis of infections in critically ill patients. Particular conditions such as community-acquired pneumonia, pediatric sepsis, or liver transplantation, among others, have been separately treated, since the optimal approaches and markers might be different in these special cases.

Advances in Clinical Chemistry, Volume 65
ISSN 0065-2423
http://dx.doi.org/10.1016/B978-0-12-800141-7.00002-4

ABBREVIATIONS

AdVs adenoviruses
AKI acute kidney injury
AUC area under the curve
BC blood culture
CAP community-acquired pneumonia
cfDNA cell-free DNA
CI confidence interval
CNS central nervous system
CRP C-reactive protein
CT-proET-1 carboxyterminal fragment of proendotelin-1
DAMPs danger-associated molecular patterns
GCS Glasgow Coma Scale
HMGB-1 high-mobility group box-1
ICU intensive care unit
IL interleukin
iNOS inducible nitric oxide synthase
MALDI matrix-assisted laser desorption ionization
MR-proADM midregional proadrenomedullin
MR-proANP medium region of pro A-type natriuretic peptide
MS mass spectrometry
NF-κB nuclear factor κB
NO nitric oxide
NSE neuron-specific enolase
OLT orthotopic liver transplantation
PAI-1 plasminogen activator inhibitor type 1
PCT procalcitonin
PRR pattern-recognition receptors
PSI pneumonia severity index
PSP/reg pancreatic stone peptide/regenerating peptide
SIRS systemic inflammatory response syndrome
ST signal transduction
TF transcription factor
TFPI tissue factor pathway inhibitor
TLRs toll-like receptors
TNF tumoral necrosis factor
TOF time of flight

1. BACKGROUND

Sepsis is the primary cause of death in the intensive care unit (ICU). Early antibiotic therapy plays a crucial role on the prognosis of these patients.

The prevention of sepsis complications requires an early and accurate diagnosis as well as the appropriate monitoring. At present, most microbiological laboratories are limited by poor sensitivity and the time-consuming nature of culture-based methods. The clinical symptoms of the septic patient are often masked by systemic inflammatory response processes, infectious or not, and treatments. The diagnostic difficulty of sepsis is even more relevant in both pediatric and adult ICUs, where patients are at increased risk because of not only their critical state and immunological vulnerability but also the use of invasive techniques (e.g., mechanical ventilation) and nonspecific symptoms. Moreover, the preventive administration of antibiotics in critical care patients increases resistance and the risk of hospital-acquired infections. On the other hand, time factor also plays an important role in the prognosis of sepsis, since a delay in the identification and appropriate management of critical patients during the first 6 h of admission to the ICU is associated with higher mortality [1].

The concept of systemic inflammatory response syndrome (SIRS) was established for the first time at the Sepsis Consensus Conference held in 1992 by the Society of Critical Care Medicine and the American College of Chest Physicians [2]. Since then, SIRS has been defined according to well-established criteria (Table 2.1), without the need for the demonstration of the presence of bacterial infection by microbiological culture, whereas sepsis is considered as a SIRS in response to documented infection that can lead to severe consequences, including multiple organ failure.

Other stages of sepsis were also defined, such as severe sepsis, associated with organ dysfunction, hypoperfusion or hypotension, and septic shock, the most severe stage of sepsis, cursing with arterial hypotension despite adequate fluid resuscitation.

The differentiation between infectious SIRS and other etiologies, as well as the stratification of the disease progression in these three stages, is very important, since their management and evolution are quite different [3].

Table 2.1 SIRS definition requires showing two or more of the following conditions

Temperature	$<36\ ^{\circ}C$ or $>38\ ^{\circ}C$
Heart rate	>90 beats/min, in absence of pain or anemia
Respiratory rate	>20 breaths/min or $PaCO_2 < 32$ mmHg
WBC count	>12,000 cells/μL or >10% immature (band) forms

However, the clinical application of these definitions quickly demonstrated that they lacked sufficient diagnostic efficiency. The presence of clinical signs of inflammation is shown in both infectious and noninfectious processes, microbiological culture being the reference differential diagnostic tool. The development of new biochemical markers of inflammation, such as C-reactive protein (CRP), procalcitonin (PCT), and interleukin-6 (IL-6), improved the differentiation of both clinical situations, thus allowing the redefinition of SIRS and sepsis in the International Sepsis Definition Conference in 2001 [4]. By this time, a new staging system was established, named PIRO system (from Predisposition, Infection, Response, and Organ Dysfunction) aimed at stratifying patients based on not only the clinical features but also the biochemical markers of inflammation (Fig. 2.1; Ref. [5]).

From a more clinical perspective, it has been recently proposed to include evidence of organ dysfunction in the criteria for sepsis [6]. Of note, this slightly differs from the definition used in the recently published guidelines of the Surviving Sepsis Campaign in which sepsis is defined clinically as the presence (probable or documented) of infection together with systemic manifestations of infection and severe sepsis is defined as sepsis with sepsis-induced organ dysfunction or tissue hypoperfusion [7].

Despite the great advance in our knowledge on the pathogenesis of sepsis, severe sepsis, defined as sepsis with organ failure, remains associated with an unacceptable high mortality [8].

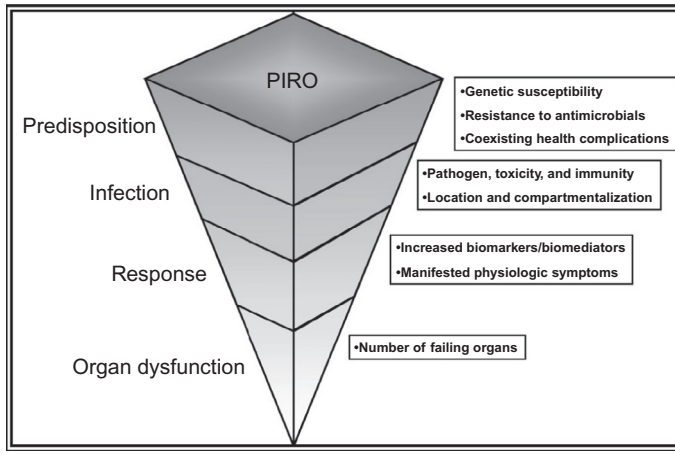

Figure 2.1 Optimum individualized treatment according to PIRO classification of patient's characteristics.

2. IMMUNOLOGIC BASIS OF SEPSIS

The inflammatory process starts as a response mediated by cellular and humoral factors that seek to limit, eliminate, and repair the lesion caused by the infectious agent. This inflammatory response is sometimes exaggerated and not limited only to the lesion point, thus spreading to the entire organism and leading to a SIRS.

Activating agents, infectious or not, will be recognized by immune system cells by surface receptors (Fig. 2.2; Ref. [9]). SIRS begins with a rapid release of proinflammatory cytokines (IL-1α, IL-1β, IL-2, IL-6, IL-8, IL-15, IL-18, and α-tumoral necrosis factor or TNF-α), as well as immunological cytokines, γ-interferon, and TNF-β. Liberation of proinflammatory cytokines is very fast, and they are rapidly cleared from systemic circulation. TNF-α plays a central role in the pathogenesis of SIRS: it is released into peripheral blood 90–120 min after the stimulus, it peaks after 2–4 h, and it returns to undetectable levels after 6 h.

PCT reaches peripheral blood several hours after proinflammatory cytokines liberation, plasma levels being increased at 3–4 h and showing the highest levels around 18 h from stimulus. The mechanism of PCT activation

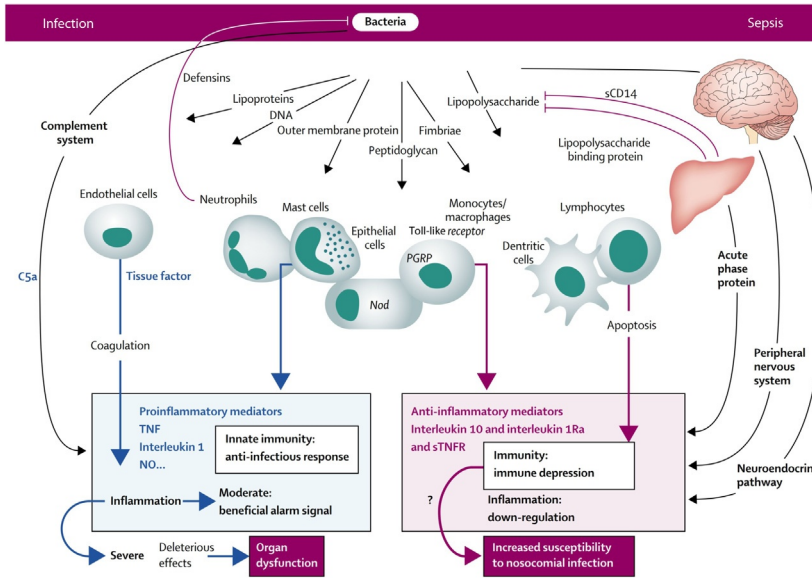

Figure 2.2 From infection to sepsis.

during infection is not completely elucidated yet, although it mainly seems to be related to TNF-α, IL-1β, and IL-6 [10,11]. Two mechanisms have been proposed for bacteria-induced PCT, direct or indirect. In the direct pathway, bacterial structural components induce an intracellular signal transduction (ST) leading to release of PCT. In the indirect pathway, bacterial ST induces mediators, such as proinflammatory cytokines, which subsequently act on the target cells to release PCT. The direct and indirect pathways can operate mutually exclusive or act simultaneously together [11].

On the other hand, the role of PCT as an active participant in the development and progression of infectious processes has been recently described [12]. At least two hypotheses have been proposed for the ubiquitous expression of PCT during infection. In the first condition (Fig. 2.3A), following infection, a specific transcription factor (TF), absent in normal situations, is activated in multiple tissues and binds to the regulatory sequences of *Calc-1* gene, inducing PCT transcription. In the second condition (Fig. 2.3B), in the normal situation, PCT transcription is actively suppressed by a specific TF that is bound to Calc-1 promoter. However, this TF is modified following infection, leading to its detachment and allowing subsequent transcription of PCT.

2.1. Innate immunity and inflammation in sepsis

Present trends lead to the consensus that sepsis results from an imbalance in the host regulation of proinflammatory SIRS and the compensatory anti-inflammatory response syndrome [5]. Sepsis initiates an inflammatory response that directly and indirectly spreads tissue damage. Pattern-recognition receptors (PRR) are the central components of the innate immune system that recognize danger signals such as invading bacteria and initiate the immune response [13]. But the host defense triggers a response from both the innate immune system and the adaptive immune system, stimulating the specific humoral cell-mediated response that amplifies the innate immunity [14]. Figure 2.4 [14] shows the unique cell wall molecules from Gram-positive and -negative bacteria, called pathogen-associated molecular PRR (toll-like receptors, TLRs) on the surface of immune cells. However, PRR can also recognize endogenous danger signals, termed alarmins or DAMPs (danger-associated molecular patterns), which are released during inflammatory stress (e.g., burns, trauma, and tissue necrosis). Examples of DAMPs that cause further amplification of the proinflammatory response through TLR-4 include heat-shock proteins,

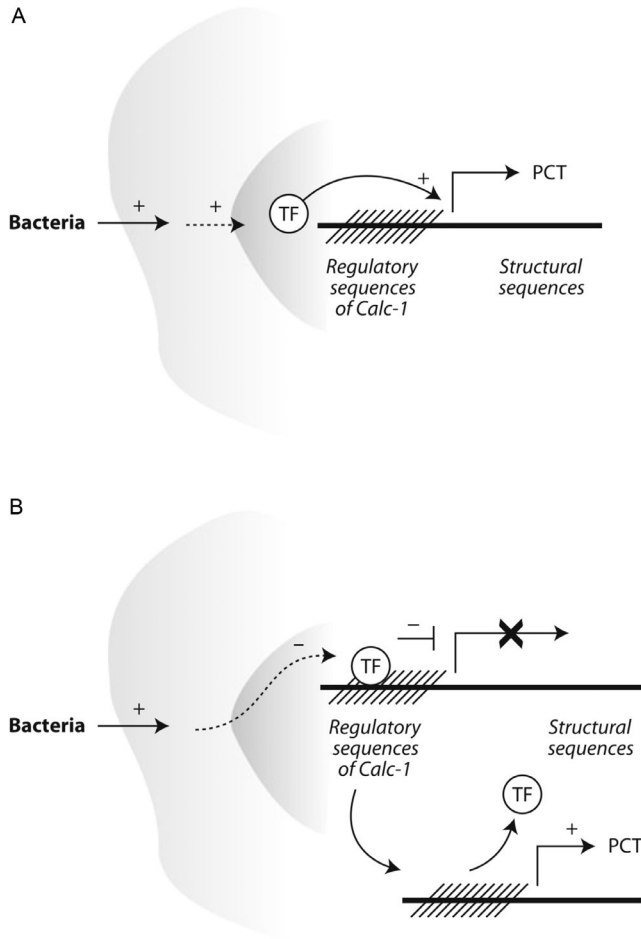

Figure 2.3 Hypotheses for the ubiquitous expression of PCT during infection.

fibrinogen, S100 proteins, hyaluronic acid, and high-mobility group box-1 (HMGB-1) protein [15].

Gram-negative lipopolysaccharides and Gram-positive peptidoglycan bind to TLR-2 and TLR-4, respectively [16]. The binding to TLR-2 and TLR-4 activates transduction pathways from intracellular signal, which lead to the activation of cytosolic nuclear factor κB (NF-κB). NF-κB migrates from cytoplasm to the nucleus, binds to transcription starting sites, and increases the transcription of cytokines, such as TNF-α, IL-1β, and IL-10. TNF-α and IL-1β are proinflammatory cytokines that not only activate the adaptive immune response but also cause direct and indirect damage

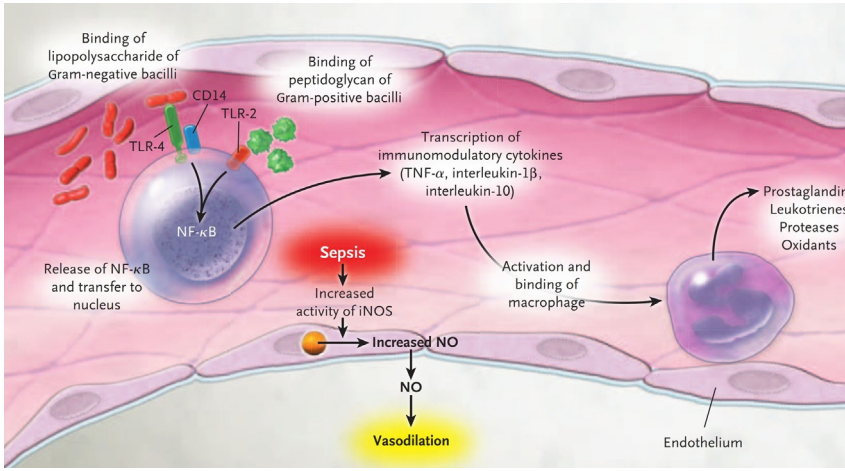

Figure 2.4 Inflammatory response to sepsis.

to the host. Proinflammatory cytokines promote the release of anti-inflammatory cytokines (IL-4 and IL-10), as well as antithrombotic mediators (C protein and antithrombin), even inducing lymphocytes apoptosis. IL-10 shows several anti-inflammatory effects and inactivates macrophages. Sepsis increases the activity of inducible nitric oxide synthase (iNOS), which increases the synthesis of nitric oxide (NO), a potent vasodilator. Cytokines activate endothelial cells regulating the adhesion receptors and endothelial damage cells by the induction of neutrophils, monocytes, macrophages, and platelets that bind to endothelial cells. These effector cells liberate mediators, such as proteases, oxidants, prostaglandins, and leukotrienes, which damage the key functions of endothelial cells producing increased permeability, with the correspondent vasodilatation and alteration of the procoagulant–anticoagulant balance. Cytokines also activate the coagulation cascade, as described in the following sections.

2.2. Procoagulant–anticoagulant equilibrium

The intricate relationship between inflammation and coagulation may have major consequences for the pathogenesis of microvascular failure and subsequent multiple organ failure, as a result of severe infection and the associated systemic inflammatory response [17]. In fact, sepsis initiates a coagulation process through endothelial activation to increase the expression of tissue factor (Fig. 2.4; Ref. [14]). The activation of the coagulation cascade, especially factors V–VIIIa, leads to the formation of α-thrombin,

which converts fibrinogen into fibrin. Fibrin binds to the platelets that adhere to endothelial cells, forming microvascular thrombi. These microvascular thrombi amplify damage through the liberation of mediators and microvascular obstruction, causing ischemia and tissue hypoxia.

Usually, the natural anticoagulants (C and S proteins), antithrombin III, and the tissue factor pathway inhibitor (TFPI) prevent coagulation, promote fibrinolysis, and eliminate microthrombi. Thrombin-α binds to thrombomodulin in the endothelial cells, significantly increasing the activation of C protein. C protein forms a complex with its cofactor, S protein. Activated C protein inactivates factors V and VIIIa through proteolysis and reduces the synthesis of plasminogen activator inhibitor type 1 (PAI-1) [18]. The protective effects of activated C protein are mediated through endothelial cell protein C receptor and protease-activated receptor 1, through the inhibition of HMGB-1 signaling [19].

On the contrary, sepsis promotes the synthesis of PAI-1, as well as a decrease in C protein, S protein, antithrombin III, and TFPI levels. Therefore, the changes induced by sepsis during the coagulation process originate increased levels of dissemination markers of intravascular coagulation and organic dysfunction [15].

3. NEW TOOLS FOR BACTERIAL IDENTIFICATION

During years, the bacterial identification at Clinical Microbiological laboratory has been routinely carried out according to microorganism phenotype (morphology, wall structure identified by different stainings, microorganism's ability to grow in different culture media, temperature, and atmosphere conditions, ability to use different biochemical routes or substrates, and, in some cases, a characteristic sensitivity to certain antimicrobials). Some of these technologies have not progressed since decades, whereas others have led to the implementation of miniaturization, automatization, and considerable better turnaround times.

However, most of the microbiological techniques remain based on principles that require growing microorganisms, with identification thus lasting from hours to weeks from sampling. On the other hand, these techniques show evident limitations related to applicability and reliability when applied to microorganisms that are difficult to grow in culture media or with limited biochemical activity.

Some years ago, different methodologies, mainly based on genetic techniques, were developed in an effort to overcome those limitations. These

approaches show important contributions in several aspects, such as reliability in the identification of the microorganism and direct diagnosis without previous culture of the sample. Several recent studies have shown that bacterial DNA load in blood directly correlates with the severity of infection and also has prognostic value [20,21].

However, hitherto, these methods failed to replace the classical ones due to technical complexity, availability limited to certain microorganisms, and the cost/benefit ratio. In fact, the repercussion of genetic testing methods as a whole has been important in clinical virology, whereas in bacteriology, mycology, and parasitology, this importance is, for the moment, much lower. As an example, although the development of an assay for detection and quantification of the adenoviruses (AdVs) with a high degree of genetic heterogeneity presented a great diagnostic challenge, a powerful new real-time quantitative polymerase chain reaction method for detection and quantification of 54 types of human AdVs has recently been reported [22]. This homogeneous technique guarantees high throughput and reduces the need for hand labor during laboratory diagnostics. The test is sensitive, inexpensive, and reproducible, and it has been extensively validated to assess the method's suitability and reliability for use in routine diagnostics.

On the other hand, the first description of mass spectrometry (MS) for bacteria identification dates from 1975 [23]. In that time, the mass range was limited to small molecules, thus restricting the application of MS to bacterial lipids. Since proteins show one major order of magnitude, softer ionization techniques were required to allow their analysis, such as matrix-assisted laser desorption ionization (MALDI). In 2004, the first full database for bacteria identification based on surface molecules analysis was described, but the high variability of these proteins limited its usefulness due to the highly rigorous standardization required. However, the new matrix allowed the ionization of proteins, mainly from ribosomal origin, making the routine identification of bacteria easier [24,25].

Recent advances in MS allow the identification of spectral patterns from serum or plasma samples with the potential to rapidly provide diagnostic signatures that can be used for disease diagnosis, prognosis, or monitoring. Kiehntopf *et al.* [26] described a reproducible pattern of plasma proteins that can discriminate between infectious and noninfectious SIRS,interestingly confirming the previous finding that a more pronounced fragmentation of α1 antitrypsin is found in sepsis samples due to the cleavage by metalloproteinases released from neutrophils at sites of inflammation [27]. However, despite some optimistic reports, the clinical validity of such patterns is still unclear and numerous issues must be addressed regarding

preanalytical, analytical, and postanalytical influences on MALDI-time of flight (TOF)-MS profiling approaches before these assays are ready for implementation in the clinical setting [28,29]. The identification of bacteria in blood cultures (BCs) by MALDI-TOF was first described several years ago, with a sensitivity ranging from 66% to 80% [30,31]. The limitations of these techniques include poor yield when there is low bacterial density, misidentification of closely related bacteria, and inability to correctly identify all pathogens in polymicrobial infections [32].

Nevertheless, MALDI-TOF-MS is expected to rapidly become a new routine resource in clinical microbiology laboratories. Its usefulness for bacterial identification is more and more generally accepted, mainly in BC [31,33,34], although there is still some reluctance as regards specific bacterial groups and some other microorganisms, such as molds. Ferreira *et al.* [35] described the direct identification by a fast method (<30 min) based on direct MALDI-TOF-MS applied to 318 BCs, in which microorganism identifications in Gram-negative coincided with conventional identification, at species level, in 83.3% of BCs and in 96.6% at the genus level. In Gram-positive, identifications coincided with conventional identification in 31.8% of BC at species level and in 64.8% at genus level. Fungemia was not reliably detected by MALDI-TOF. In 18 BCs positive for *Candida* species (8—*C. albicans*, 9—*C. parapsilosis*, and 1—*C. tropicalis*), no microorganisms were identified at species level, and only one (5.6%) was detected at genus level.

There are other potential applications of this technology in clinical microbiology, which are beginning to be explored and may become a reality in the near future, such as epidemiologic studies, identification of pathogenicity factors, and antimicrobial resistance studies [36].

4. USEFUL MARKERS OF INFECTIOUS COMPLICATIONS ACCORDING TO THE AFFECTED ORGAN AND PATIENT STATUS

Different biochemical markers have also been proposed to guide the correct management of potential complications in those critically ill patients under suspicion or risk of infection. Some interesting findings are reviewed in the following sections.

4.1. Central nervous system complications in septic patients

Central nervous system (CNS) complications frequently occur in septic patients before failure of other organs. Septic encephalopathy can be

explained as a disruption of normal CNS function through changes in metabolism, alterations in cell signaling through inflammatory mediators, and aberrant regulatory function exerted by the brain over the body. Diagnostically, septic encephalopathy is not explained by other factors frequently found in septic patients, such as metabolic abnormalities, and it is often difficult to evaluate in the ICU setting, where most of these patients are treated and in which patients are often medically sedated [37]. Clinical scores such as the Glasgow Coma Scale (GCS), confusion assessment method for the ICU (CAM-ICU), or the assessment to intensive care environment (ATICE) are helpful in establishing a diagnosis of delirium/encephalopathy and can be predictive clinical markers for the mortality rate of the ongoing central septic process. Nevertheless, a number of serum markers such as S100β, neuron-specific enolase (NSE), and glial fibrillary acidic protein can serve as general markers of brain injury that may provide helpful information in sedated patients. Hsu *et al.* [38] described that S100β levels more closely reflected severe encephalopathy and type of brain lesions than NSE and GCS, but the usefulness of S100β levels in evaluating encephalopathy in septic patients has been questioned by other authors [39]. In sepsis, leukocytes are activated, they adhere to the blood vessel and move into the tissue, a process mediated by adhesion molecules, whose expression is increased in septic encephalopathy, whereas platelet endothelial cell adhesion molecule remains unaltered [40]. Hypoxic-ischemic brain injury can be the final common pathway of different insults to the brain. The discussed finding of hypoxic-ischemic lesions in patients with sepsis is therefore in itself a non-specific change, but changes in microcirculation are associated with the upregulation of inflammatory gene transcripts including TNF-α, IL-1β, and iNOS. In contrast to these early changes, generalized brain edema through the disruption of the blood brain barrier may occur later and not as an initial early event in the disruption of normal circulation. Nevertheless, research focused on these pathogenesis paths has mainly been carried out in animal models, thus requiring further studies to zone in on crucial markers that will allow a definite prognosis in clinical practice [37].

4.2. Renal failure during sepsis

Development of acute kidney injury (AKI) during sepsis increases patient morbidity, predicts higher mortality, and is associated with an increased length of stay in the ICU. Interestingly, the pathophysiology of septic AKI requires a different approach than AKI of nonseptic origin [41]. For

instance, the excretion of IL-18 is higher in septic than in nonseptic AKI, allowing to predict deteriorating kidney function approximately 24–48 h before clinically significant AKI [42]. AKI can be diagnosed by small changes in serum creatinine or acute reductions in urine output. Nevertheless, rising serum creatinine or oliguria during sepsis often appears after the window of opportunity for effective therapy has already passed. Moreover, sepsis reduces the production of creatinine without major alterations in body weight, hematocrit, or extracellular fluid and creates further limitations on using changes in creatinine levels as a reliable marker of AKI. Therefore, clinicians must pay attention to several emerging markers that are being validated for early detection of AKI, aimed at guiding appropriate and timely interventions in septic AKI (Cystatin C [43], L-type acid-binding protein [44], neutrophil gelatinase-associated lipocalin [45], kidney injury molecule-1 [46], and netrin-1 [47]).

4.3. Infection severity after trauma or surgery

Trauma is the leading cause of death during the first four decades of life in developed countries. Classically, death caused by trauma follows a trimodal distribution: immediate, early, and late. The late group typically occurs in the ICU several days after trauma because of septic complications and multiple organ failure. The early detection of infectious complications through the application of suitable biomarkers may have its greatest effect on improved outcomes following severe trauma [48]. In polytrauma patients, the current leading candidate biomarker of sepsis is PCT, since PCT levels are intimately related to the presence (or absence) of bacterial endotoxin in the circulation, and moreover, PCT spikes shortly after major traumatic injury. The magnitude of this level is correlated to the propensity to develop multiple organ failure [49].

Postsurgical complications cause a high level of morbidity and mortality. As in other severe bacterial infections, the prognosis varies according to how early the diagnosis is made and whether a correct treatment against the etiologic agent is used. During the postoperative period, intensive monitoring of patients on antibacterial treatment is important to avoid a delay in a change of therapy in case it is inadequate. Serial measurements of CRP are widely used, but in postoperative patients, this protocol has many limitations. One of the main disadvantages is the increase expected in CRP concentrations over the 20–40 days following surgery. Thus, in most cases, CRP does not offer an adequate guide for the early treatment of infection

after surgery [50]. In fact, abdominal surgery increases CRP in almost all patients during the first postoperative period [51,52].

Therefore, PCT constitutes an alternative marker for trauma and post-surgical monitoring, since it differentiates between a local infectious process, that can be treated by surgical reintervention, and a systemic one, which would need a change in antibiotic therapy rather than an unnecessary surgical approach [53], even helping to predict the outcome in critically ill patients [54]. Moreover, PCT shows a favorable kinetic profile for use as a clinical marker: it promptly increases within 6–12 h upon stimulation and circulating PCT levels halve daily when the infection is controlled by the host immune system or antibiotic therapy [55]. PCT correlates with bacterial load [56,57] and severity of infection [58]. On the other hand, in a study carried out by Novotny *et al.* in a cohort of 160 patients diagnosed with sepsis after abdominal surgery, a multivariate analysis revealed that APACHE II score and PCT are early independent markers in predicting death by sepsis. The combination of both parameters in a simple mathematical formula made possible to calculate a prognosis score with high diagnostic efficiency [59]. At the single-test level, the assessment of PCT is a suitable alternative for the calculation of the APACHE score, since it was found to be equally valuable for prognosis prediction.

For upper and lower respiratory tract infection in ICU patients with sepsis and postoperative infections, randomized-controlled studies have also shown the efficacy of using PCT algorithms to guide antibiotic decisions. For other types of infections, only observational studies are available which are importantly limited by the lack of a true gold standard [60].

Other gut-derived substances have been identified and have many similarities to PCT both in terms of the predictive ability as a biomarker and in the pattern of release following traumatic injury. Pancreatic stone peptide/regenerating peptide (PSP/reg) is a lectin-binding acute-phase protein whose release is promoted by IL-6. Once in the bloodstream, PSP/reg causes the activation of leukocytes and has been shown to be a sensitive indicator of sepsis. In a study comparing PCT with PSP/reg in moderately to severely injured trauma patients, PSP/reg seemed to be a better sign of infectious complications in the confusing hyperinflammatory postinjury state [61].

4.4. Liver transplantation and infections

The main complications of orthotopic liver transplantation (OLT) are early hepatic dysfunction, infection, sepsis, and rejection [62]. The way and

degree in which such complications can modify the final success of a transplantation are still unclear [63,64].

Acute rejections and infections usually appear within the first weeks after transplantation, and their similar clinical findings make differential diagnosis difficult. The first sign of a potential rejection is usually a nonspecific alteration of hepatic markers. Infection is the most frequent complication, being associated with a high posttransplant mortality rate [65].

The early identification of an acute rejection, and its differential diagnosis from infection, is still a clinical challenge in OLT. Several studies conducted with patients who underwent heart, lung, kidney, or liver transplantation have indicated that PCT plasma concentrations increase during infection but not in rejection. This biochemical marker thus seems to play an important role in the differential diagnosis of both clinical processes [66,67], as well as prognosis [68]. The origin of posttransplantation PCT induction remains unclear. The most widely accepted explanation is that both proinflammatory cytokines release and bacterial endotoxin contamination from the bowel flora during surgery are responsible for post-OLT increase of PCT concentration [69,70]. This fact could also be related to ischemic-perfusion organ damage during surgery, as well as a partial hepatic origin of PCT, recently described not only as a marker but also as a mediator of infection [12].

Other new biomarkers have been studied as a potential aid to the follow-up of OLT patients, such as the carboxyterminal fragment of proendotelin-1 (CT-proET-1), the midregional proadrenomedullin (MR-proADM), and the medium region of pro A-type natriuretic peptide (MR-proANP). Our group described the reference change value of these three biomarkers in post-OLT patients, the lowest one being found for CT-proET-1 (90%), followed by MR-proADM (112%) and MR-proANP (127%). The same work showed that in the first postoperative control, MR-proADM was the most efficient marker—even better than PCT—in predicting severe complications, a cut-off point of 2.5 nmol/L being selected according to the ROC analysis [71]. Local and nonsevere infections showed much lower concentrations, usually below 0.5 nmol/L.

4.5. Infections in critically ill pediatric patients

Clinical and diagnostics predictors of serious disease in a child with fever are still being explored for early diagnosis so that therapy could be appropriately targeted. Host susceptibility and immune response are making strides in the understanding of disease pathogenesis and in identifying the causes of fever.

In critically ill children, PCT is better than CRP for differentiating bacterial and nonbacterial SIRS, and the diagnostic accuracy is enhanced by combining these tests with clinical judgement [72]. PCT and IL-6 positively correlate with the pediatric risk of mortality score, and their diagnostic value has been demonstrated in pediatric sepsis [73]. Moreover, PCT has also been described as a useful tool not only in diagnosing sepsis [74,75], but also in stratifying severity [76,77], and in predicting the outcome after cardiac arrest in pediatric ICUs [78].

However, controversial results have been found regarding the neonatal period of life, especially in preterm, very low weight infants, and infants with comorbidities and prolonged hospitalization. Conventionally, the most accepted classification considers early-onset neonatal sepsis when the diagnosis is made within the first week, typically the first 72 h of life, whereas late-onset neonatal sepsis takes place beyond the first week [32]. In both cases, the diagnosis is challenging for several reasons, mainly related to the low performance of BC. A low density and intermittent bacteremia, as well as small volume of blood samples [79], are common, and additionally, infants and/or mothers may have received antibiotics prior to BC. A recent meta-analysis of 29 studies using PCT in the diagnosis of neonatal sepsis described a pooled sensitivity of 81% (95% confidence interval, CI: 74–87%) with a pooled specificity of 79% (95% CI: 69–87%) and an area under the curve (AUC) of 0.87. On stratifying the meta-analysis into early-onset and late-onset neonatal sepsis, the AUC was higher for the latter (0.95) as compared to the former (0.78), thus suggesting better accuracy of PCT in diagnosis of late-onset neonatal sepsis. However, the authors draw attention to the high statistical heterogeneity found in the works, so these results must be interpreted with caution [80].

IL-6 is an emergent and promising new marker for the neonatal period of life, since the development of fully automated and reliable methods with turnaround times adequate to an emergency laboratory [81]. Although ILs such as IL-6 are not specific to a particular disease or infection, they are reasonably accurate in positively identifying neonatal infection since, apart from sepsis, there are relatively few inflammatory conditions that will substantially influence their circulating levels in term and preterm infants. The measurement of IL-6 in cord blood adds interest to its potential application as a noninvasive diagnostic method [81]. However, these proteins fail to differentiate sepsis and other pathologies such as necrotizing enterocolitis, which involves different management strategies and long-term outcomes [82]. It does not provide either precise information on the type of cell damaged or the exact location of injury.

Thus, in recent years, investigators have been focused on the discovery of novel biomarkers [83] for more precise identification of specific diseases or specific organ pathologies. In this context, the use of metagenomics for molecular identification of a vast array of microorganisms allows, for instance, high-throughput 16S rRNA sequencing to be used to obtain specific bacterial sequence signatures in stool samples. This technique may potentially be transformed into an efficient diagnostic tool or a prophylactic measure, but there is no universal agreement on the best methodological approach and cost [32]. On the other hand, intracellular proteins released in response to cell injury, such as DAMPs, have been described as useful tools in early-onset sepsis (within the first 72 h of life), wherein the interface between the maternal and fetal compartments is compromised by inflammation, leading to translocation of DAMPs into the fetus [84]. The same research group identified a specific amniotic fluid proteomic fingerprint of intraamniotic inflammation: the "mass restricted score," ranging from 0 to 4, based on the absence or presence of four biomarkers: neutrophil defensin 1, neutrophil defensin 2, S100A12, and S100A8 [85].

4.6. Early recognition of severe sepsis in community-acquired pneumonia

The assessment of community-acquired pneumonia (CAP) severity is a cornerstone in its management, facilitating the selection of the most appropriate care site and empirical antibiotic therapy. However, there are significant limitations to the use of clinical judgement alone to assess CAP severity [86], and a range of clinical scoring systems have therefore been developed. The pneumonia severity index (PSI) is a scoring system based on 20 variables (demographic and clinical together with associated comorbidities). Patients are stratified into five classes according to the 30-day risk of death; three have a low risk of mortality at 30 days (0.1–2.8%), a fourth has an increased risk (4–10%), and a fifth has a high risk (27%). It is suggested that patients with PSI scores I–II can be treated as outpatients, whereas those in classes IV and V should be admitted for inpatient treatment. PSI performs well as a predictor of mortality in CAP (AUC: 0.70–0.89), but only moderately well as a predictor of hospital/ICU admission (AUC: 0.56–0.85) [87].

The British Thoracic Society proposed the use of CURB65, to assess CAP severity [88]. This is a 5-point scoring system resulting in three categories associated with different 30-days mortality rates (low risk: 0.7–0.2%, intermediate risk: 13%, and high risk: 17–57%). When compared with PSI,

CURB65 has a similar discriminatory ability to predict mortality (AUC: 0.73–0.87) but is worse at predicting ICU admission (AUC: 0.6–0.78) [87].

This scoring system has the advantage over PSI that it is easier to calculate, favoring its routine implementation, and it does not directly address comorbidities. Some limitations are the underestimation of the risk in elderly patients with underlying diseases and that it relies on laboratory data, limiting its use outside the hospital setting.

Other usual scores are SMART-COP, a tool designed to predict patients that will require intensive respiratory or vasopressor support, with a higher discriminatory ability than PSI or CURB65 [89], and the severe CAP score, based on two major criteria and six minor criteria. This score outperforms both PSI and CURB65 in the prediction of adverse outcome (ICU admission, need for mechanical ventilation, severe sepsis, and treatment failure) [90].

There is growing interest in the use of biomarkers to assess CAP severity, since many of them can help to predict bacteremia (e.g., PCT) [91] and mortality (e.g., PCT, D-dimer, cortisol, copeptin, natriuretic peptides, and proADM) [92,93]. In a long-term survival study including 1740 patients with proven CAP, MR-proANP and copeptin levels increased with increasing severity of CAP, classified according to CURB65 score. In patients who died within 28 and 180 days, median MR-proANP (313.9 vs. 80.0 and 277.8 vs. 76.0 pmol/L, each $p < 0.0001$) and copeptin (42.6 vs. 11.2 and 33.2 vs. 10.7 pmol/L, each $p < 0.0001$) levels were significantly higher than the levels in survivors. In receiver-operating characteristics analysis for survival at 28 and 180 days, the AUCs for copeptin (0.84; 95% CI: 0.82–0.86, and 0.78; 95% CI: 0.76–0.80) and MR-proANP (0.81; 95% CI: 0.79–0.83, and 0.81; 95% CI: 0.79–0.83) were superior to the AUC of CURB65 (0.74; 95% CI: 0.71–0.76, and 0.71; 95% CI: 0.69–0.74, $p < 0.05$), PCT, CRP, and white blood cells. In multivariable Cox proportional-hazards regression analyses adjusted for comorbidity and pneumonia severity, MR-proANP and copeptin were independent and the strongest predictors of short-term and long-term mortality [94].

Levels of MR-proADM, the most stable fragment of adrenomedullin, increase in sepsis [95] and correlate well with PSI and mortality (short and long term) [96,97]. Its addition to clinical scoring systems significantly improves their discriminatory power [98], and it is useful in the prediction of serious complications in CAP patients.

Several other biomarkers, such as glycation end-products [99], HMGB-1 [100], and soluble triggering receptor expressed on myeloid cell-1 [101],

have been evaluated, but there are no data yet to support their widespread use in the assessment of CAP severity.

4.7. Cell-free DNA as a potential marker of infection

With the recent developments in the study of circulating nucleic acids, its application in different diagnostic fields has increased. The usefulness of the quantification of cell-free DNA (cfDNA) concentration in the diagnosis of infections in febrile patients and as a prognostic marker in septic patients has already been described [102]. High plasma cfDNA concentrations have been shown to be associated with sepsis outcome, showing a moderate discriminative power regarding ICU mortality, but not hospital mortality [103], and nonsurvivors expressing apoptotic DNA fragmentation bands [104]. These findings support the conception that apoptosis plays a critical role in the pathogenesis of sepsis. Thus, cfDNA could be used as a noninvasive, rapid, sensitive, and accurate marker of severe bacteremia, although clinical value in severe sepsis and septic shock as well as the origin of cfDNA remain unclear.

In trauma patients, however, the sensitivity of plasma cfDNA as a potential prognosis marker especially death, after severe injury, presents limitations for clinical use. No evidence was found to relate plasma cfDNA to infection in these patients. Circulating cfDNA seems to be connected with injury type and mode, open wounds, and surgical operations, which may be the primary reasons for the observed plasma cfDNA increase [105].

5. CONCLUSIONS

Different biochemical markers have been proposed to guide the correct management of potential complications in those critically ill patients under suspicion or risk of infection. PCT can be shown as one of the most interesting biomarkers, with proven usefulness in pediatric and adult ICUs as well as after severe trauma or surgery, including OLT. Promising results have also been obtained with other new markers, such as MR-proADM, both in CAP and OLT, or IL-6 in the neonatal period of life. In addition, several emerging markers are being validated for early detection of AKI, aimed at guiding appropriate and timely interventions in septic AKI. Finally, further studies will be needed to prove the S100 ability to detect brain injury in septic patients or the potential prognostic value of high plasma cfDNA concentrations in severe infections.

REFERENCES

[1] E.P. Rivers, L. McIntyre, D.C. Morro, K.K. Rivers, Early and innovative interventions for severe sepsis and septic shock: taking advantage of a window of opportunity, CMAJ 173 (9) (2005) 1054–1065.

[2] American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference Committee, Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis, Crit. Care Med. 20 (1992) 864–874.

[3] C. Mitaka, Clinical laboratory differentiation of infectious versus non-infectious systemic inflammatory response syndrome, Clin. Chim. Acta 351 (2005) 17–29.

[4] M.M. Levy, M.P. Fink, J.C. Marshall, E. Abraham, D. Angus, D. Cook, et al., 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference, Crit. Care Med. 31 (2003) 1250–1256.

[5] S.D. Carrigan, G. Scott, M. Tabrizian, Toward resolving the challenges of sepsis diagnosis, Clin. Chem. 50 (2004) 1301–1314.

[6] J.L. Vincent, S.M. Opal, J.C. Marshall, K.J. Tracey, Sepsis definitions: time for change, Lancet 381 (2013) 774–775.

[7] R.P. Dellinger, M.M. Levy, A. Rhodes, D. Annane, H. Gerlach, S.M. Opal, Surviving Sepsis Campaign Guidelines Committee including the Pediatric Subgroup, et al., Surviving sepsis campaign: international guidelines for management of severe sepsis and septic shock: 2012, Crit. Care Med. 41 (2013) 580–637.

[8] D.F. Gaieski, J.M. Edwards, M.J. Kallan, B.G. Carr, Benchmarking the incidence and mortality of severe sepsis in the United States, Crit. Care Med. 41 (2013) 1167–1174.

[9] D. Annane, E. Bellisant, J.M. Cavaillon, Septic shock, Lancet 365 (2005) 63–78.

[10] H. Redl, G. Schlag, E. Togel, M. Assicot, C. Bohuon, Procalcitonin release patterns in a baboon model of trauma and sepsis: relationship to cytokines and neopterin, Crit. Care Med. 28 (2000) 3659–3663.

[11] K.T. Whang, S.D. Vath, K.L. Becker, R.H. Snider, E.S. Nylen, B. Muller, et al., Procalcitonin and proinflammatory cytokine interactions in sepsis, Shock 14 (2000) 73–78.

[12] G.N. Matwiyoff, J.D. Prahl, R.J. Miller, J.J. Carmichael, D.E. Amundson, G. Seda, et al., Immune regulation of procalcitonin: a biomarker and mediator of infection, Inflamm. Res. 61 (2012) 401–409.

[13] K. Schroder, J. Tschopp, The inflammasomes, Cell 140 (2010) 821–832.

[14] J.A. Russell, Management of sepsis, N. Engl. J. Med. 355 (2006) 1699–1713.

[15] W.J. Wiersinga, S.J. Leopold, D.R. Cranendonk, T. van der Poll, Host innate immune responses to sepsis, Virulence (2013). http://dx.doi.org/10.4161/viru.25436.

[16] S.M. Opal, C.E. Huber, Bench-to-bedside review: toll-like receptors and their role in septic shock, Crit. Care 6 (2002) 125–136.

[17] M. Levi, The coagulant response in sepsis and inflammation, Hamostaseologie 30 (2010) 10–16.

[18] M. Levi, T. van der Poll, Inflammation and coagulation, Crit. Care Med. 38 (2010) S26–S34.

[19] J.S. Bae, A.R. Rezaie, Activated protein C inhibits high mobility group box 1 signaling in endothelial cells, Blood 118 (2011) 3952–3959.

[20] R.P. Peters, R.F. de Boer, T. Schuuman, S. Gieverld, M. Kooistra-Smid, M.A. van Aqtmael, et al., *Streptococcus pneumonia* DNA load in blood as a marker of infection in patients with community-acquired pneumonia, J. Clin. Microbiol. 47 (2009) 3308–3312.

[21] Y.C. Ho, S.C. Chang, S.R. Lin, W.K. Wang, High levels of mecA DNA detected by quantitative real-time PCR assay are associated with mortality in patients with

methicillin-resistant *Staphylococcus aureus* bacteremia, J. Clin. Microbiol. 47 (2009) 1443–1451.
[22] I. Bil-Lula, N. De Franceschi, K. Pawlik, M. Woźniak, Improved real-time PCR assay for detection and quantification of all 54 known types of human adenoviruses in clinical samples, Med. Sci. Monit. 18 (6) (2012) BR221–BR228.
[23] J.P. Anhalt, C. Fenselau, Identification of bacteria using mass spectrometry, Anal. Chem. 47 (1975) 219–225.
[24] J.M. Hettick, M.L. Kashon, J.P. Simpson, P.D. Siegel, G.H. Mazurek, D.N. Weissman, Proteomic profiling of intact mycobacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Anal. Chem. 76 (2004) 5769–5776.
[25] S. Rupf, K. Breitung, W. Schellenberger, K. Merte, S. Kneist, K. Eschrich, Differentiation of mutans streptococci by intact cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Oral Microbiol. Immunol. 20 (2005) 267–273.
[26] M. Kiehntopf, D. Schmerler, F.M. Brunkhorst, R. Winkler, K. Ludewig, D. Osterloh, et al., Mass spectrometry-based protein patterns in the diagnosis of sepsis/systemic inflammatory response syndrome, Shock 36 (2011) 560–569.
[27] M.C. Vissers, P.M. George, J.C. Bathurst, S.O. Brennan, C.C. Winterbourn, Cleavage and inactivation of alpha 1-antitrypsin by metalloproteinases released from neutrophils, J. Clin. Invest. 82 (1988) 706–711.
[28] M. Kiehntopf, R. Siegmund, T. Deufel, Use of SELDI-TOF mass spectrometry for identification of new biomarkers: potential and limitations, Clin. Chem. Lab. Med. 45 (2007) 1435–1449.
[29] P. Seng, M. Drancourt, F. Gouriet, B. La Scola, P.E. Fournier, J.M. Rolain, et al., Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry, Clin. Infect. Dis. 49 (2009) 543–551.
[30] B. La Scola, D. Raoult, Direct identification of bacteria in positive blood culture bottles by matrix-assisted laser desorption ionization time-of-flight mass spectrometry, PLoS One 4 (2009) e8041.
[31] L.G. Stevenson, S.K. Drake, P.R. Murray, Rapid identification of bacteria in positive blood culture broths by matrix-assisted laser desorption ionization-time of flight mass spectrometry, J. Clin. Microbiol. 48 (2010) 444–447.
[32] L. Srinivasan, M.C. Harris, New technologies for the rapid diagnosis of neonatal sepsis, Curr. Opin. Pediatr. 24 (2012) 165–171.
[33] A. Ferroni, S. Suárez, J.L. Beretti, B. Dauphin, E. Bille, J. Meyer, et al., Real-time identification of bacteria and *Candida* species in positive blood culture broths by matrix-assisted laser desorption ionization-time of flight mass spectrometry, J. Clin. Microbiol. 48 (2010) 1542–1548.
[34] B. La Scola, Intact cell MALDI-TOF mass spectrometry-based approaches for the diagnosis of bloodstream infections, Expert Rev. Mol. Diagn. 11 (2011) 287–298.
[35] L. Ferreira, F. Sánchez-Juanes, F. Porras-Guerra, M.I. García-García, J.E. García-Sánchez, J.M. González-Buitrago, et al., Microorganisms direct identification from blood culture by matrix assisted laser desorption/ionization time-of-flight mass spectrometry, Clin. Microbiol. Infect. 17 (2011) 546–551.
[36] J.L. Muñoz, S. Vega, L. Ferreira, F. Sánchez, J.M. González-Buitrago, Aplicaciones de la proteómica en el laboratorio de Microbiología Clínica, Enferm. Infecc. Microbiol. Clin. 30 (7) (2012) 383–393.
[37] P. Pytel, J.J. Alexander, Pathogenesis of septic encephalopathy, Curr. Opin. Neurol. 22 (2009) 283–287.

[38] A.A. Hsu, K. Fenton, S. Weinstein, J. Carpenter, H. Dalton, M.J. Bell, Neurological injury markers in children with septic shock, Pediatr. Crit. Care Med. 9 (2008) 245–251.
[39] O. Plazza, E. Russo, S. Cotena, G. Esposito, R. Tufano, Elevated S100β levels do not correlate with the severity of encephalopathy during sepsis, Br. J. Anaesth. 99 (2007) 518–521.
[40] S. Hofer, C. Bopp, C. Hoerner, K. Plaschke, R.M. Faden, E. Martin, et al., Injury of the blood brain barrier and upregulation of ICAM-1 in polymicrobial sepsis, J. Surg. Res. 146 (2008) 276–281.
[41] A. Zajou, A. Agarwal, Sepsis and acute kidney injury, J. Am. Soc. Nephrol. 22 (2011) 999–1006.
[42] S.M. Bagshaw, C. Langenberg, M. Haase, L. Wan, C.N. May, R. Bellomo, Urinary biomarkers in septic cute kidney injury, Intensive Care Med. 33 (2007) 1285–1296.
[43] P. Devarajan, Proteomics for the investigation of acute kidney injury, Contrib. Nephrol. 160 (2008) 1–16.
[44] T. Nakamura, T. Sugaya, H. Koide, Urinary liver-type fatty acid-binding protein in septic shock: effect of polymyxin B-immobilized fiber hemoperfusion, Shock 31 (2009) 454–459.
[45] D.S. Wheeler, P. Devarajan, Q. Ma, K. Harmon, M. Monaco, N. Cvijanovich, et al., Serum neutrophil gelatinase-associated lipocalin (NGAL) as a marker of acute kidney injury in critically ill children with septic shock, Crit. Care Med. 36 (2008) 1297–1303.
[46] K. Doi, A. Leelahavanichkul, P.S. Yuen, R.A. Star, Animal models of sepsis and sepsis-induced kidney injury, J. Clin. Invest. 119 (2009) 2868–2878.
[47] G. Armes, C.D. Krawczeski, J.G. Woo, Y. Wang, P. Devarajan, Urinary netrin-1 is an early predictive biomarker of acute kidney injury after cardiac surgery, Clin. J. Am. Soc. Nephrol. 5 (2010) 395–401.
[48] C.A. Adams, Sepsis biomarkers in polytrauma patients, Crit. Care Clin. 27 (2011) 345–354.
[49] T. Hensler, S. Sauerland, R. Lefering, M. Nagelschmidt, B. Bouillon, J. Andermahr, et al., The clinical value of procalcitonin and neopterin in predicting sepsis and organ failure after major trauma, Shock 20 (2003) 420–426.
[50] M. Lindberg, A. Hole, H. Johnsen, A. Asberg, A. Rydning, H.E. Myrvold, et al., Reference intervals for procalcitonin and C-reactive protein after major abdominal surgery, Scand. J. Clin. Lab. Invest. 62 (2002) 189–194.
[51] H.B. Reith, U. Mittelkotter, E.S. Debus, C. Kussner, A. Thiede, Procalcitonin in early detection of postoperative complications, Dig. Surg. 15 (1998) 260–265.
[52] M. Meisner, K. Tschaikowsky, A. Hutzler, C. Schick, J. Schuttler, Postoperative plasma concentrations of procalcitonin after different types of surgery, Intensive Care Med. 24 (1998) 680–684.
[53] P. Svoboda, I. Kantorova, P. Scheer, J. Radvanova, M. Radvan, Can procalcitonin help us in timing of re-intervention in septic patients after multiple trauma or major surgery? Hepatogastroenterology 54 (2007) 359–363.
[54] A.A. Dahaba, B. Hagara, A. Fall, P.H. Rehak, W.F. List, H. Metzler, Procalcitonin for early prediction of survival outcome in postoperative critically ill patients with severe sepsis, Br. J. Anaesth. 97 (2006) 503–508.
[55] K.L. Becker, Procalcitonin and the calcitonin gene family of peptides in inflammation, infection, and sepsis: a journey from calcitonin back to its precursors, J. Clin. Endocrinol. Metab. 89 (2004) 1512–1525.
[56] P. Schuetz, B. Mueller, A. Trampuz, Serum procalcitonin for discrimination of blood contamination from bloodstream infection due to coagulase-negative staphylococci, Infection 35 (2007) 352–355.

[57] C. van Nieuwkoop, T.N. Bonten, J.W. van't Wout, E.J. Kuijper, G.H. Groeneveld, M.J. Becker, et al., Procalcitonin reflects bacteremia and bacterial load in urosepsis syndrome: a prospective observational study, Crit. Care 14 (2010) R206.
[58] P. Schuetz, M. Christ-Crain, W. Albrich, W. Zimmerli, B. Mueller, Guidance of antibiotic therapy with procalcitonin in lower respiratory tract infections: insights into the ProHOSP study, Virulence 1 (2010) 88–92.
[59] A. Novotny, K. Emmanuel, E. Matevossian, M. Kriner, K. Ulm, H. Bartels, et al., Use of procalcitonin for early prediction of lethal outcome of postoperative sepsis, Am. J. Surg. 194 (2007) 35–39.
[60] P. Schuetz, W. Albrich, B. Mueller, Procalcitonin for diagnosis of infection and guide to antibiotic decisions: past, present and future, BMC Med. 9 (2011) 107.
[61] M. Keel, L. Harter, T. Reding, L.K. Sun, M. Hersberger, B. Seifert, et al., Pancreatic stone protein is highly increased during posttraumatic sepsis and activates neutrophil granulocytes, Crit. Care Med. 37 (2009) 1642–1648.
[62] P.D. Russ, D.E. Elliot, J.D. Durham, K. Garg, M.E. Wasch, I. Kam, Liver transplantation complications, eMed. J. (2007). http://emedicine.com/radio/topic883.htm#section
[63] D. Kunz, M. Pross, W. Konig, H. Lippert, T. Manger, Diagnostic relevance of procalcitonin, IL-6 and cellular immune status in the early phase after liver transplantation, Transplant. Proc. 30 (1998) 2398–2399.
[64] R. Moreno, M. Berenguer, Post-liver transplantation medical complications, Ann. Hepatol. 5 (2006) 77–85.
[65] M.C. Warle, H.J. Metselaar, W.C. Hop, I.C. Gyssens, M. Kap, J. Kwekkeboom, et al., Early differentiation between rejection and infection in liver transplant patients by serum and biliary cytokine patterns, Transplantation 75 (2003) 146–151.
[66] M. Staehler, C. Hammer, B. Meiser, B. Reichart, Procalcitonin: a new marker for differential diagnosis of acute rejection and bacterial infection in heart transplantation, Transplant. Proc. 29 (1997) 584–585.
[67] S. Hammer, F. Meisner, P. Dirschedl, G. Hobel, P. Fraunberger, B. Meiser, et al., Procalcitonin: a new marker for diagnosis of acute rejection and bacterial infection in patients after heart and lung transplantation, Transpl. Immunol. 6 (1998) 235–241.
[68] B. Prieto, E. Llorente, I. González-Pinto, F.V. Álvarez, Plasma procalcitonin measured by time-resolved amplified cryptate emission (TRACE) in liver transplant patients. A prognosis marker of early infectious and non-infectious postoperative complications, Clin. Chem. Lab. Med. 46 (2008) 660–666.
[69] S. Hammer, F. Meisner, C. Hammer, D. Seidel, Use of procalcitonin as indicator of nonviral infections in transplantation and related immunologic diseases, Transplant. Rev. 14 (2000) 52–63.
[70] M. Kretzschmar, A. Kruger, W. Schirrmeister, Procalcitonin following elective partial liver resection—origin from the liver? Acta Anaesthesiol. Scand. 45 (2001) 1162–1167.
[71] D. Miguel, B. Prieto, F.V. Álvarez, Biological variation and prognosis usefulness of new biomarkers in liver transplantation, Clin. Chem. Lab. Med. 51 (6) (2013) 1241–1249.
[72] A. Rampersad, D. Mukundan, Fever, Curr. Opin. Pediatr. 21 (2009) 139–144.
[73] J.R. Fioretto, J.G. Martin, C.S. Kurokawa, M.F. Carpi, R.C. Bonatto, S.M. Ricchetti, et al., Interleukin-6 and procalcitonin in children with sepsis and septic shock, Cytokine 43 (2008) 160–164.
[74] C. Clec'h, J.P. Fosse, P. Karoubi, F. Vincent, I. Chouahi, L. Hamza, et al., Differential diagnostic value of procalcitonin in surgical and medical patients with septic shock, Crit. Care Med. 34 (2006) 102–107.
[75] F. Leclerc, R. Cremer, O. Noizet, Procalcitonin as a diagnostic and prognostic biomarker of sepsis in critically ill children, Pediatr. Crit. Care Med. 4 (2003) 264–266.

[76] E.D. Carrol, P. Newland, A.P. Thomson, C.A. Hart, Prognostic value of procalcitonin in children with meningococcal sepsis, Crit. Care Med. 33 (2005) 224–225.
[77] C. Rey, M. Los Arcos, A. Concha, A. Medina, S. Prieto, P. Martinez, et al., Procalcitonin and C-reactive protein as markers of systemic inflammatory response syndrome severity in critically ill children, Intensive Care Med. 33 (2007) 477–484.
[78] M. Los Arcos, C. Rey, A. Concha, A. Medina, B. Prieto, Acute-phase reactants after paediatric cardiac arrest. Procalcitonin as marker of immediate outcome, BMC Pediatr. 8 (2008) 18, http://dx.doi.org/10.1186/1471-2431-8-18.
[79] T.G. Connell, M. Rele, D. Cowley, J.P. Buttery, N. Curtis, How reliable is a negative blood culture result? Volume of blood submitted for culture in routine practice in a children's hospital, Pediatrics 119 (2009) 891–896.
[80] E.K. Vouloumanou, E. Plessa, D.E. Karageorgopoulos, E. Mantadakis, M.E. Falagas, Serum procalcitonin as a diagnostic marker for neonatal sepsis: a systematic review and meta-analysis, Intensive Care Med. 37 (2011) 747–762.
[81] B. Prieto, D. Miguel, M. Costa, D. Coto, F.V. Álvarez, New quantitative electrochemiluminescence method (ECLIA) for interleukin-6 (IL-6) measurement, Clin. Chem. Lab. Med. 48 (6) (2010) 835–838.
[82] H.S. Lam, S.P. Wong, H.M. Cheung, W.C. Chu, R.P. Wong, K.M. Chui, et al., Early diagnosis of intra-abdominal inflammation and sepsis by neutrophil CD64 expression in newborns, Neonatology 99 (2011) 118–124.
[83] P.C. Ng, H.S. Lam, Biomarkers in neonatology: the next generation of tests, Neonatology 102 (2012) 145–151.
[84] C.S. Buhimschi, M.A. Baumbucsh, A.T. Dulay, E.A. Oliver, S. Lee, G. Zhao, et al., Characterization of RAGE, HMGB1 and S100beta in inflammation-induced preterm birth and fetal tissue injury, Am. J. Pathol. 175 (2009) 958–975.
[85] C.S. Buhimschi, I.A. Buhimschi, S. Abdel-Razeq, V.A. Rosenberg, S.F. Thung, G. Zhao, et al., Proteomic biomarkers of intra-amniotic inflammation: relationship with funisitis and early-onset sepsis in the premature neonate, Pediatr. Res. 61 (2007) 318–324.
[86] A. Singanayagam, J.D. Chalmers, A.T. Hill, Severity assessment in community acquired pneumonia: a review, QJM 102 (2009) 379–388.
[87] J.D. Chalmers, P. Mandal, A. Singanayagam, A.R. Akram, G. Choudhury, P.M. Short, et al., Severity assessment tools to guide ICU admission in community-acquired pneumonia: systematic review and meta-analysis, Intensive Care Med. 37 (2011) 1409–1420.
[88] W.S. Lim, S.V. Baudouin, R.C. George, A.T. Hill, C. Jamieeson, I. Le Jeune, et al., BTS guidelines for the management of community-acquired pneumonia in adults: update 2009, Thorax 64 (2009) iii1–iii55.
[89] P.G.P. Charles, R. Wolfe, M. Whitby, M.J. Fine, A.J. Fuller, R. Stirling, et al., SMART-COP: a tool for predicting the need for intensive respiratory or vasopressor support in community-acquired pneumonia, Clin. Infect. Dis. 47 (2008) 375–384.
[90] P.P. Yandiola, A. Capelastegui, J. Quintana, R. Díez, I. Gorodo, A. Bilbao, et al., Prospective comparison of severity scores for predicting clinically relevant outcomes for patients hospitalized with community-acquired pneumonia, Chest 135 (2009) 1572–1579.
[91] F. Muller, M. Christ-Crain, T. Bregenzer, M. Krause, W. Zimmerli, B. Mueller, et al., Procalcitonin levels predict bacteremia in patients with community-acquired pneumonia: a prospective cohort trial, Chest 138 (2010) 121–129.
[92] P. Schuetz, I. Suter-Widmer, A. Chaudri, M. Christ-Crain, W. Zimmerli, B. Mueller, Prognostic value of procalcitonin in community-acquired pneumonia, Eur. Respir. J. 37 (2011) 384–392.

[93] J.M. Pereira, J.A. Paiva, J. Rello, Severe sepsis in community-acquired pneumonia—early recognition and treatment, Eur. J. Intern. Med. 23 (2012) 412–419.
[94] S. Krüger, S. Ewig, J. Kunde, O. Hartmann, N. Suttorp, T. Welte, The CAPNETZ Study Group, Pro-atrial natriuretic peptide and pro-vasopressin for predicting short-term and long-term survival in community-acquired pneumonia: results from the German Competence Network CAPNETZ, Thorax 65 (2010) 208–214.
[95] M. Christ-Crain, N.G. Morgenthaler, J. Struck, S. Harbarth, A. Bergmann, B. Müller, Mid-regional pro-adrenomedullin as a prognostic marker in sepsis: an observational study, Crit. Care 9 (2005) 816–824.
[96] M. Christ-Crain, N.G. Morgenthaler, D. Stolz, C. Müller, R. Bingisser, S. Harbarth, et al., Pro-adrenomedullin to predict severity and outcome in community-acquired pneumonia, Crit. Care 10 (2006) R96–R103.
[97] D.T. Huang, D.C. Angus, N.A. Pugh, J.A. Kellum, L.A. Weissfeld, R.L. Delude, et al., Proadrenomedullin in emergency department patients with community-acquired pneumonia, Ann. Emerg. Med. 52 (2008) S41.
[98] P. Schuetz, M. Wolbers, M. Christ-Crain, R. Thomman, C. Falconnier, I. Widmer, et al., Prohormones for prediction of adverse medical outcome in community-acquired pneumonia and lower respiratory tract infections, Crit. Care 14 (2010) R106.
[99] T. Uchida, M. Shirasawa, L.B. Ware, K. Kojima, Y. Hata, K. Makita, et al., Receptor for advanced glycation endproducts is a marker of type I cell injury in acute lung injury, Am. J. Respir. Crit. Care Med. 173 (2006) 1008–1015.
[100] D.C. Angus, L. Yang, L. Kong, J.A. Kellum, K.L. Delude, K.J. Tracey, et al., Circulating high-mobility group box 1 (HMGB1) concentrations are elevated in both uncomplicated pneumonia and pneumonia with severe sepsis, Crit. Care Med. 35 (2007) 1061–1067.
[101] S. Gibot, A. Cravoisy, B. Levy, M.C. Bene, G. Faure, P.E. Bollaert, Soluble triggering receptor expressed on myeloid cells and the diagnosis of pneumonia, N. Engl. J. Med. 350 (2004) 451–458.
[102] V.G. Moreira, B. Prieto, J.S. Rodríguez, F.V. Álvarez, Usefulness of cell-free plasma DNA, procalcitonin and C-reactive protein as markers of infection in febrile patients, Ann. Clin. Biochem. 47 (2010) 253–258.
[103] K. Saukkonen, P. Lakkisto, V. Pettila, M. Varpula, S. Karlsson, E. Ruokonen, For the Finn sepsis Study Group, et al., Cell-free plasma DNA as a predictor of outcome in severe sepsis and septic shock, Clin. Chem. 54 (6) (2008) 1000–1007.
[104] R. Huttunen, T. Kuparinen, J. Jylhävä, J. Aittoniemi, R. Vuento, H. Huhtala, et al., Fatal outcome in bacteremia is characterized by high plasma cell free DNA concentration and apoptotic DNA fragmentation: a prospective cohort study, PLoS One 6 (7) (2011) e21700, http://dx.doi.org/10.1371/journal.pone.0021700.
[105] B. Ren, F. Liu, F. Xu, J. He, H. Zhu, G. Zou, Is plasma cell-free DNA really a useful marker for diagnosis and treatment of trauma patients? Clin. Chim. Acta 424 (23) (2013) 109–113.

CHAPTER THREE

Metabolomics in Nephrotoxicity

Ying-Yong Zhao[*,1], **Rui-Chao Lin**[†,1]
[*]Key Laboratory of Resource Biology and Biotechnology in Western China, Ministry of Education, The College of Life Sciences, Northwest University, Xi'an, Shaanxi, PR China
[†]School of Chinese Materia Medica, Beijing University of Chinese Medicine, Beijing, PR China
[1]Corresponding authors: e-mail address: zyy@nwu.edu.cn; zhaoyybr@163.com; linrch307@sina.com

Contents

Abstract

Nephrotoxicity or renal toxicity can be a result of hemodynamic changes, direct injury to cells and tissue, inflammatory tissue injury, and/or obstruction of renal excretion. Nephrotoxicity is frequently induced by a wide spectrum of therapeutic drugs and environmental pollutants. Knowledge of the complex molecular and pathophysiologic mechanisms leading to nephrotoxicity remains limited, in part, by research that historically focused on single or relatively few risk markers. As such, current kidney injury biomarkers are inadequate in terms of sensitivity and specificity. In contrast, metabolomics enables screening of a vast array of metabolites simultaneously using NMR and MS to assess their role in nephrotoxicity development and progression. A more comprehensive understanding of these biochemical pathways would also provide valuable insight to disease mechanisms critical for drug development and treatment.

Advances in Clinical Chemistry, Volume 65
ISSN 0065-2423
http://dx.doi.org/10.1016/B978-0-12-800141-7.00003-6

1. INTRODUCTION

Metabolomics has shown great potential in the identification of early biochemical changes associated with nephrotoxicity, as well as, the discovery of potential biomarkers, and underlying disease mechanisms. Metabolomics, as a system approach, could provide extensive information on the dynamic process of nephrotoxicity from drug toxicity or environmental pollutants. The aim of this chapter is to review the metabolomic approach for biomarker discovery in the field of nephrotoxicity to provide an overview of the contribution of metabolomics to nephrotoxicity research and how this approach has advanced our understanding of this disease process. Through this review, researchers will better understand the potential of these technologies and will gain additional knowledge on the advantages and applications of this approach in nephrotoxicity. The perspectives of metabolomics for nephrotoxicity are then discussed.

2. NEPHROTOXICITY OR RENAL TOXICITY

The nephron, comprised of glomerulus and renal tubule, is the functional unit of the kidney. The three main functions of the kidneys include plasma filtration and maintenance of whole-body electrolyte homeostasis, elimination, and concentration of waste products from both endogenous metabolism and exogenous metabolism, and synthesis and secretion of hormones (rennin and erythropoietin). Renal blood flow is approximately 25% of resting cardiac output. The glomerular, tubular, and renal interstitial cells frequently encounter significant concentrations of endogenous (protein, drug–protein binding, and their metabolites) and exogenous metabolites (medications and their metabolites) that can induce changes in kidney function and structure. As such, the kidney is at risk due to exposure to high volumes of blood-borne endogenous and exogenous metabolites including toxicants and toxic metabolites [1]. Nephrotoxicity or renal toxicity can be a result of hemodynamic changes, direct injury to cells and tissue, inflammatory tissue injury, and/or obstruction of renal excretion. Nephrotoxicity is frequently induced by a wide spectrum of therapeutic drugs (antibiotic, immunosuppressant, antineoplastic agent, nonsteroidal anti-inflammatory drugs, heroin, cocaine, and natural medicines) and environmental pollutants (heavy metals, organic solvents, insecticides, and glycols).

According to anatomical location and onset time for clinical symptoms, nephrotoxicity is divided into several subcategories including acute renal

injury (ARI), acute tubular necrosis, acute nephritis, nephritic syndrome, chronic renal insufficiency, etc. ARI due to nephrotoxicity can be subdivided into tubular, glomerular, interstitial, and vascular damage. Each type has partly distinct injury pattern and clinical course [2]. Acute interstitial injury is an allergic response that causes a strong inflammatory response. However, tubular injury is frequently a direct toxic injury to epithelial tubules and lacks inflammation. Less common injury patterns include nephrocalcinosis, osmotic renal injury, and crystal nephropathy [3]. The drug-induced renal injury involved acute or chronic renal injury. Acute tubular necrosis is usually drug's dose-dependent toxicity (aminoglycoside, cisplatin, and amphotericin B) [3]. Chronic renal injury is frequently caused by prolonged high-dose exposure to certain analgesics and immunosuppressants [3]. Additionally, chronic exposure to environmental pollutants can lead to chronic renal injury.

Nephrotoxicity leads to detrimental effects in the kidney, which clinically become evident as decreased renal function. The interest in nephrotoxicity has grown rapidly in the decade. The number of serious adverse effects is growing faster than the number of prescriptions. Nephrotoxicity is the major reason that a drug is removed from markets. Current biomarkers (blood urea nitrogen (BUN) and serum creatinine (Scr), hematoxylin–eosin staining and immunohistochemistry) for nephrotoxicity have remained unchanged for several decades. These biomarkers often lack sensitivity and specificity, partly because conventional research tools have hampered investigators by restricting their focus to a single or relatively few risk markers at one time. For example, clinical trials typically assess BUN and Scr to monitor ARI. Unfortunately, these markers do not change appreciably until significant renal injury has occurred. Lack of an efficient and accurate biomarker has become a leading cause of severe nephrotoxicity. Therefore, there is need for novel and specific biomarkers that will reduce the expenditure associated with drug discovery and development. Metabolomics plays an important role in providing novel and specific biomarkers for renal injury and for predicting nephrotoxicity. Metabolomics has been utilized to identify new biomarkers of nephrotoxicity and to better understand mechanisms of drug toxicity.

3. METABOLOMICS IN BIOMARKER DISCOVERY

Metabolomics/metabonomics is defined as the "quantitative measurement of the dynamic multi-parametric metabolic responses of living systems to pathophysiological stimuli or genetic modifications" [4]. Metabolomics is

the latest systems biology technology for understanding complex biological processes. Genomics, transcriptomics, proteomics, and metabolomics are related to the genome (DNA), the transcriptome (RNA), proteome (proteins), and metabolome (metabolites), respectively. They use a similar comprehensive approach to analyzing and identifying biological changes. Metabolomics is a global biochemical approach for biomarker discovery and provides insight into metabolic profiling across a wide range of biochemical pathways in response to a perturbation (disease, drug, and toxin). The metabolome is the measurement of small molecules from a biology system which basically covers various small molecules, including amino acids, sugars, lipids, organic acids, nucleotides, small peptides, and xenobiotics. The endogenous metabolites are the final product of interactions between gene expression, protein expression, and the cellular environment. Thus, metabolomic information complements data obtained from genomics, transcriptomics, and proteomics-adding a final piece to a systems approach for the study of disease diagnosis, biomarker discovery, and drug action [5]. Compared to traditional biochemical approaches that often focus on one or several metabolites, metabolomic approach includes quantitative data on a series of metabolites and aims at obtaining a whole understanding of metabolism related to conditions of disease and drug exposure.

Most metabolomic approaches are based on proton nuclear magnetic resonance (^{1}H NMR) spectroscopy and mass spectrometry (MS) methodologies. ^{1}H NMR-based metabolomic approach provides information on the chemical structure by determining the chemical shift of each proton of compounds. ^{1}H NMR spectra from biological samples included many peaks and each peak represents a proton in a different chemical environment. The biofluids generate a spectrum displaying overlapping and crowed peaks, but the complex peaks can be resolved using data-processing software. In addition, sample preparation of ^{1}H NMR approach is simple. However, ^{1}H NMR-based metabolomic approach is restricted to a limited number of high-concentration metabolites. An alternative approach is to use MS-based metabolomics, which requires separation of individual metabolites by either gas chromatography (GC) or liquid chromatography (LC), but offers greater sensitivity as compared to ^{1}H NMR. Thus, GC-MS or LC-MS can detect low-concentration metabolites as well as identify them accurately. However, some metabolites elude identification by mass alone due to analytical limitations in mass resolution or shared exact mass. This problem may be exacerbated in complex biological fluids. Therefore, it is important to recognize that ^{1}H NMR spectroscopy and MS are

complementary tools and that a combination of methods provides a wide range of metabolites and thus more comprehensive metabolome profile than any single approach. It is noteworthy that NMR or MS metabolomics has been increasingly used in nephrotoxicity studies [6–11].

4. METABOLOMICS APPLICATION IN ANIMAL MODEL-BASED NEPHROTOXICITY

The kidney is one of the highly susceptible organs to toxicants such as antibiotics, anticancer agents, and heavy metals. Many toxicants interfere with its function, excretion of body wastes, and maintenance of body fluid. Metabolomics has been applied to nephrotoxicity to identify novel specific biomarkers for detection and monitoring of this complex disease.

4.1. Antibiotics

Drug-induced nephrotoxicity is a major issue due to their urinary excretion [3,12,13]. To address this issue, a combination of GC-MS and LC-MS urinary and kidney metabolomics was performed in gentamicin, cisplatin, or tobramycin-induced nephrotoxicity [14]. Identified metabolites included amino acid, peptide, lipid, nucleotide, carbohydrate, energy, vitamin, and xenobiotic metabolites. Traditional histology and chemistry measurements were performed to correlate these changes with renal injury. In cisplatin-treated rats, increased BUN and Scr were found in all females and in one male at 28 days. Histopathologic kidney injury was, however, observed for all three drugs after 28 days. Urine polyamines, amino acids, glycylproline, glucosamine, 1,5-anhydroglucitol monoethanolamine, and phosphate showed an early response. Early response biomarkers were sorbitol, glucose, and 5-methyltetrahydrofolate in tissue. Increased urinary amino acids, dipeptides and sugars, and decreased tissue amino acids were caused by the three drugs. Prior to histopathologically evident kidney injury, excretion was remarkable 1–5 days, consistent with increased amino acid and sugar excretion typically associated with the nephrotoxic state [15,16]. Several urinary polyamines were increased in drug-treated rats and the increase was most significant after 1 day. Polyamines are directly involved in cell proliferation and viability and as such can reflect altered protein synthesis and breakdown [17,18]. Significantly decreased purine and pyrimidine nucleosides (inosine, 2′-deoxyinosine, adenosine, guanosine, cytidine, and uridine) were observed in tissue. These findings may reflect changes in activity of renal transporters, synthesis/breakdown of nucleotides, or filtration rates.

This panel of biomarkers may provide a noninvasive method to detect injury before histologically evident damage. This study showed that the excretion of polyamines, amino acids, sugars, inositol, monoethanolamine, and phosphate was remarkably increased before histologic and BUN/Scr abnormalities. New early nephrotoxicity biomarkers included polyamines, 1,5-anhydroglucitol, monoethanolamine, phosphate, glycylproline, glucosamine, sorbitol, and 5-methyltetrahydrofolate. This study showed that some of the well-known nephrotoxic biomarkers (amino acids and glucose) can be very early indicators of kidney malfunction.

Gentamicin, an aminoglycoside antibiotic, causes nephrotoxicity in 10–15% of cases. A combination of ^{1}H NMR and GC-MS metabolomics was used to identify novel early urinary biomarkers in gentamicin-induced nephropathy. Metabolites were linked to gut microflora and increased urinary glucose consistent with damage to the S1 and S2 proximal tubules, that is, primary sites for glucose resorption. Monitoring excretion of urinary protein provided further support for lipocalin-2 and Kim-1 as sensitive noninvasive biomarkers of nephrotoxicity [19]. Another study showed increased urinary glucose and decreased trimethylamine *N*-oxide (TMAO) by ^{1}H NMR with decreased xanthurenic and kynurenic acids and altered sulfation patterns by MS [20]. Other results showed increased urinary 6-hydroxymelatonin that directly correlated with histopathologic findings [21]. 6-Hydroxymelatonin is an oxidized by-product of melatonin, a powerful antioxidant that acts as an NO scavenger [22]. Melatonin supplementation has also been shown to protect the rat kidney from gentamicin and $HgCl_2$-induced nephrotoxicity [23,24]. Urinary 6-hydroxymelatonin excretion may therefore reflect increased melatonin as protection against gentamicin-induced kidney damage. Another study showed that tyrosine, valine, and hydroxyproline reflected histopathologic changes of kidney injury development. Glucosuria was noted much earlier than changes in the traditional markers (BUN and Scr). Glucosuria has been shown to be a potential biomarker of nephrotoxicity [15,20,25]. Dopamine-related compounds (homovanillic acid sulfate (HVA-SO_4) and homoveratric acid sulfate (HVrA-SO_4)) were significantly increased at early time points and could be early indicators of a renal adaptive response to gentamicin-induced renal injury. Metabolomics successfully identified valine, hydroxyproline, HVA-SO_4, and HVrA-SO_4 as early injury or adaptive biomarkers [26,27].

Oxytetracycline-induced nephrotoxicity was investigated in rats [28]. Oxytetracycline resulted in significantly increased serum BUN and Scr and reduced creatinine clearance. Significantly increased lipid peroxidation

biomarkers (thiobarbituric acid-reactive substances and lipid hydroperoxide) and decreased antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase), and low-molecular weight antioxidants (vitamin C, vitamin E, and reduced glutathione) levels were also observed. Oxidative injury of the cortex and medulla represented the major biochemical pathway of oxytetracycline-induced nephrotoxicity.

4.2. Immunosuppressive drugs

Commonly used immunosuppressive drugs are cyclosporine (CsA), tacrolimus, and sirolimus. CsA is a first-line immunosuppressant employed for management of solid organ transplantation and autoimmune disease. Increased urinary glucose, acetate, trimethylamine, and succinate along with reduced urinary TMAO by ^{1}H NMR and decreased kynurenic acid, xanthurenic acid, citric acid, and riboflavin by LC-MS were correlated with onset of nephrotoxicity [29]. Another study showed that the significantly increased kidney weight, Scr, BUN, cholesterol, and triglyceride were observed in CsA-induced nephrotoxicity rats. In CsA-treated control rats, tissue showed significantly increased total nitrate/nitrite concentration and malondialdehyde production as well as decreased glutathione and glutathione peroxidase activity. Histopathologic evaluation of CsA-induced control rats revealed tubular atrophy, hyaline casts, and focal tubular necrosis [30]. Reactive oxygen species played an important role in CsA-induced nephrotoxicity. Kidney tissue lipid peroxidation was significantly increased in CsA-induced nephrotoxicity [31].

Klawitter's study established a comprehensive approach including proteomics, metabolomics, histomorphology, plasma kidney function parameters, glomerular filtration rates, the urinary oxidative stress marker 15-F_{2t}-isoprostane, and blood and kidney immunosuppressant concentration to gain more detailed mechanistic insights into nephrotoxicity of CsA in combination with sirolimus [32,33]. Representative examples are increased kidney aminoacylase expression, upregulation of pyruvate dehydrogenase and fructose-1,6-bisphosphatase, and downregulation of arginine/glycine amidinotransferase [32]. Decreased glomerular filtration rate was observed in CsA- and sirolimus-treated rats at 28 days. Histology revealed tubular damage in CsA-treated rats that was enhanced with sirolimus [34]. Sirolimus enhanced CsA-induced glutathione deletion and increased blood TMAO, glucose, and cholesterol [35]. ^{1}H NMR-based urinary metabolomics revealed time-dependent changes of 2-oxoglutarate,

citrate, and succinate. In combination with increased urine isoprostane, these changes indicated oxidative stress. After 28 days of CsA treatment, urine metabolomics shifted to patterns typical for proximal tubular damage with reduction of Krebs cycle intermediates and TMAO, whereas acetate, lactate, trimethylamine, and glucose increased. Again, sirolimus enhanced these negative effects [33].

The metabolomics of CsA alone and in combination with either sirolimus or everolimus were investigated to determine whether combination therapy altered CsA nephrotoxicity [35]. Increased glucose, hydroxybutyrate, creatine, creatinine, TMAO, and cholesterol and decreased glutathione were observed in CsA-induced rats. Altered metabolites were related to increased energy metabolism via glycolysis and oxidative stress response. Metabolic profiling indicated that coadministration of everolimus ameliorated toxicity whereas sirolimus enhanced it.

Low-salt diet and CsA nephrotoxicity were also evaluated using ^{1}H NMR metabolomics in Klawitter's research group [36]. The study showed that proximal tubular injury was associated with increased urinary acetate, lactate, glucose, amino acids and decreased hippurate, creatinine, 2-oxoglutarate, succinate, and citrate. Changes in urine metabolite patterns observed after 28 days of treatment with CsA in the normal salt diet matched those described for nephrotoxins causing tubular injury. These findings corroborated histologic studies that also showed specific tubular damage [37]. In addition, this reflected urine metabolite patterns that appeared more sensitive than traditional BUN and Scr markers. Metabolic profiling in urine may provide the basis for the development of toxicodynamic monitoring strategies for immunosuppressant nephrotoxicity. In addition, CsA induced mitochondrial disturbances and activation of the Nrf2-oxidative-damage and protein-response pathways [38].

Tacrolimus (FK-56) is another calcineurin inhibitor used for immunosuppression to prevent graft rejection. Similar to CsA, tacrolimus-induced renal injury occurs by increasing oxygen free radical formation and oxidative stress [39]. Metabolomics has been applied to amphotericin B and cephaloridine nephrotoxicity studies [40,41].

4.3. Chemotherapeutics

Nephrotoxicity is one of the most important complications of treatment with anticancer drugs, for example, doxorubicin, cisplatin, ifosfamide, and platinum. Nephrotoxicity of doxorubicin was evaluated by ^{1}H

NMR. Early increased TMAO indicated renal dysfunction. Perturbation in TMAO was maximal in the low-dose group (48 h postdose), but recovered by 168 h. Increased urinary glucose, lactate, alanine, and valine suggested progression of renal injury resulting in glycosuria, lactic aciduria, and aminoaciduria in the high-dose group at 168 h. Impaired energy metabolism was evident by decreased urine TCA intermediates in rats treated with high-dose doxorubicin [42]. Changed urinary and kidney tissue metabolites were sufficiently sensitive for detection of streptozotocin-induced nephrotoxicity [43]. The major urinary biomarkers were creatine, ascorbate, succinate, lactate, citrate, allantoin, 2-ketoglutarate, and 3-hydrobutyrate. Succinate, creatine, myo-inositol, alanine, lactate, ATP, 3-hydrobutyrate, and glucose were kidney tissue markers. Metabolic changes implied enhanced lipid and ketone body synthesis and decreased TCA and glycolysis. These metabolic energy changes were associated with the pathogenicity of diabetic nephropathy. In addition, ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) metabolomics was applied to exogenous metabolite identification such as cyclophosphamide and ifosfamide [44].

Cisplatin, an antineoplastic agent to treat solid tumors, induces severe tubular and mild glomerular toxicity. Metabolomics showed altered urinary glucose, amino acids, and TCA intermediates that preceded Scr in cisplatin-induced acute renal failure [15]. An ^{1}H NMR-based urinary metabolomics study showed that 1,3-dimethylurate, taurine, glucose, glycine, and branched-chain amino acid (isoleucine, leucine, and valine) were remarkably increased and that phenylacetylglycine and sarcosine levels were decreased in the cisplatin-induced rats [45]. Another ^{1}H NMR metabolomic study was conducted on urine samples collected in 1, 2, 3, and 4 weeks. A total of 36 metabolites were increased including 1-methylnicotinamide, cytosine, 2-oxoglutarate, 3-indoxylsulfate, homocysteine, taurine, choline, creatinine, glucose, hippurate, creatine, and tryptophan. Interestingly, metabolites propionate, ethanol, and acetate were remarkably decreased [46]. Cisplatin-induced nephrotoxicity was performed in cisplatin-induced HK-2 cells. *N*-acetylasparate, creatine, uracil, proline, and acetate were decreased in HK-2 cell lysates, but lactate, acetate, and glutamate were increased in the conditioned media. Interestingly, only one metabolite, acetate, was common to both. Acetyl-CoA synthetase (AceCS2) and Sirt3 expression were markedly decreased in cisplatin-induced HK-2 cells [47]. Cisplatin-induced nephrotoxicity was also studied for different age rats by ^{1}H NMR metabolomics [48]. Increased Kim-1

provided early evidence of cisplatin-induced nephrotoxicity in the most sensitive group (80 days of age). Kim-1 tissue mRNA and urinary protein were significantly correlated to each other and to severity of histopathologic lesions.

Mitochondrial bioenergetics, redox state, and oxidative stress were investigated in cisplatin-induced nephrotoxicity [49]. Increased Scr and BUN were observed in cisplatin-induced ARI rats. Mitochondrial dysfunction was evident by decreased mitochondrial membrane electrochemical potential and calcium uptake. Interestingly, the NO synthase inhibitor, 2-amino-4-methylpridine, enhanced nephrotoxicity [50]. Overall, these results clearly supported a central role for mitochondria in cisplatin-induced nephrotoxicity.

4.4. Natural medicines

Aristolochic acid (AA), widely present in *Aristolochia*, *Bragantia*, and *Asarum* species, is generally believed to cause acute renal failure, characterized by interstitial fibrosis, hyperproteinemia, severe anemia, and uremia [51]. Metabolomics is a promising approach for rapid screening of nephrotoxicity associated with ingestion of multi-ingredient medicinal herb supplements. It also provides a valid method for exploring chemical-induced perturbations in the metabolic and lesion network. Several papers have been published for evaluation of the AA nephrotoxicity using metabolomics. A combined GC-MS and LC-MS metabolic profiling was designed to evaluate AA nephrotoxicity. In this study, histopathologic findings were associated with systemic disturbances in the metabolic network involving free fatty acids, energy and amino acid metabolism and alterations in the structure of gut microbiota [52]. A subsequent study showed that homocysteine formation and the folate cycle were significantly increased, but metabolic pathways of AA biosynthesis were decreased in these rats [53]. Kynurenic acid, hippuric acid, citric acid, and glucuronide-containing metabolites in urine and glucuronide conjugate, bile acids, lysophosphatidylcholines (lysoPCs) and fatty acids in plasma were observed as potential biomarkers [54–57]. Significantly increased Scr was also observed in the AA-induced rats, which indicated that AA induced an adverse effect on renal clearance. AA nephropathy exhibited early proximal tubular injury and disordered fatty acid metabolism. Increased plasma BUN and kidney tissue fatty acids and triglycerides were observed in AA-treated rats. These findings accompanied downregulation of renal rOAT1/3, rOCT1/2, and rOCTN1/2 expression. Specifically,

OCTN2 transported L-carnitine through the cell membrane. AA also induced significantly decreased L-carnitine in the renal cortex. Downregulation of peroxisome proliferator-activated receptor alpha (rPPARα) and carnitine acyltransferase 1 (rCPT1), and upregulation of acetyl coenzyme A carboxylase 1/2 (rACC1/2) in the renal cortex were detected [58]. AA caused proximal tubular and papillary lesions with the former suffering the main damage. Nephrotoxicity was dose-dependent during onset, development, and partial recovery [59,60]. These results were also supported by the conventional biochemical parameters.

Morning glory seed (MGS) is widely used for the treatment of edema, ascites, simple obesity, and lung fever. Long-term MGS exposure can result in progressive renal injury. In recent years, UPLC-MS metabolomics was employed to characterize the metabolic profile of plasma, urine, and kidney tissue extracts of MGS-induced nephrotoxicity. Results indicated that specific metabolic pathways, that is, lysoPCs formation and phenylalanine biosynthesis, were decreased [61]. Changes in amino acids, citric acid, creatinine, cholic acid, and 5-methyltetrahydrofolate were observed in rat urine [62]. Metabolomics has also been used to identify kidney tissue biomarkers [63]. *Rhizoma alismatis* is widely used for diuretic, anti-nephrolithic, hypolipidemic, antiatherosclerotic, antidiabetic, and anti-inflammatory purposes. UPLC-MS metabolomics was used to study the nephrotoxicity of *R. alismatis* in rats. Using this approach, 13 urinary biomarkers were detected. The method effectively discriminated treated rats at 60, 120, and 180 days with serious organic damage apparent (day 180) by histology [64]. In addition, metabolomics has characterized nephrotoxicity of cinnabar, realgar, and Zhusha Anshen Wan [65–67]. As can be seen, metabolomics provides a powerful approach to diagnose and more fully understand nephrotoxicity associated with natural medicines.

4.5. Environmental pollutants

Many heavy metals, such as lead, mercury, cadmium, and uranium, are potentially nephrotoxic. Most metals cause nephrotoxicity via damage to the proximal tubules cells even at relatively low exposure. Lead and cadmium can alter the normal absorptive function of proximal tubule cells and inhibit their mitochondrial function. This leads to significantly decreased oxidative phosphorylation and ATP in tubular epithelium cells followed by accumulation of glucose, amino acids, and protein in urine [68,69].

A procedure for ^{1}H NMR urinary metabolomics was proposed to evaluate nephrotoxicity induced by $HgCl_2$ [70]. In treated rats, glucose, acetate, alanine, lactate, succinate, and ethanol were significantly increased, whereas 2-oxoglutarate, allantoin, citrate, formate, taurine, and hippurate were significantly decreased. These endogenous metabolites were selected as putative biomarkers for $HgCl_2$-induced nephrotoxicity. Changes in lactate, acetate, succinate, and ethanol were dose dependent. In contrast, 2-oxoglutarate, citrate, formate, glucose, and taurine were significantly changed only at higher $HgCl_2$. Another urinary metabolomics study showed increased lactate, alanine, acetate, succinate, trimethylamine, and glucose as well as decreased citrate and α-ketoglutarate by ^{1}H NMR and LC-MS in $HgCl_2$-induced rats. Kynurenic acid, xanthurenic acid, pantothenic acid, 7-methylguanine, phenol sulfate, and benzene diol sulfate were decreased [25]. ^{1}H NMR metabolomics showed relatively higher concentrations of valine, isobutyrate, threonine, and glutamate in $HgCl_2$-treated rats. Increased glutamate was present in the renal cortex and may be associated with $HgCl_2$-induced renal acidosis and disruption of the TCA cycle [71]. Nephrotoxicity of iron oxides, $CdCl_2$, nanosized copper particle, and lithium was evaluated using metabolomics [72–75].

4.6. Melamine

Melamine poisoning has become widely publicized after a recent exposure of infants and children to melamine-tainted milk in China. Renal damage resulted from kidney stones formed from melamine, uric acid, and cyanuric acid. Urinary metabolomics was performed to evaluate the global biochemical alteration triggered by melamine ingestion in parallel with ARI in rats [76]. The results indicated that melamine caused serious nephrotoxicity in perturbed amino acid metabolism including tryptophan, polyamine, and tyrosine metabolism, altered TCA cycle, and gut microflora structure. Cyanuric acid, produced in the gut by microbial transformation of melamine, served as an integral stone component responsible for melamine-induced renal toxicity in rats. It should be noted that melamine toxicity was attenuated and excretion increased after antibiotic suppression of gut microbial activity [77]. Analysis of urinary amino acids showed that hydroxyproline was increased in a manner consistent with chemical and histopathologic findings. Furthermore, the highest urinary hydroxyproline concentration was found in rats with kidney fibrosis. Males were slightly more affected than females, a finding supported by hydroxyproline

excretion. As such, hydroxyproline may be a noninvasive urinary biomarker for detection of ARI associated with kidney fibrosis [78]. Another ^{1}N NMR metabolomics study showed increased asparagine, choline, creatinine, cysteine, ethanolamine, glucose, isoleucine, glutamine, and myo-inositol in medullar tissues, but glucitol, phenylalanine, tyrosine, and *sn*-glycero-3 were decreased. Ethanolamine, hypoxanthine, isoleucine, and O-phosphoethanolamine were increased in the renal cortex, whereas formate, glucose, glutathione, threonine, and myo-inositol were decreased [79]. Histologic analysis revealed epithelial degeneration and necrotic cell death in the proximal and distal tubules.

4.7. Other nephrotoxicity

LC-MS and GC-MS were used to identify small molecule biomarkers of ARI that could aid in a better mechanistic understanding of SCH 900424-induced ARI in mice. Metabolomics revealed that 3-indoxyl sulfate was a more sensitive marker (vs. Scr or BUN) of renal toxicity. Plasma 3-indoxyl sulfate was markedly increased. Significantly decreased urinary 3-indoxyl sulfate excretion may be due to renal toxicity associated with its increased plasma concentration. In addition, *p*-cresol sulfate, kynurenate, kynurenine, and 3-indole lactate might be important biomarkers [80]. To evaluate untargeted metabolic profiling as a tool for gaining insight into underlying nephrotoxicity, Fischer 344 male rats were dosed with fenofibrate (300 mg/kg/day) for 14 days and urine and plasma were analyzed on days 2 and 14 [81]. Reduced TCA intermediates and biochemical evidence of lactic acidosis demonstrated that energy metabolism homeostasis was altered. Perturbation of glutathione biosynthesis and increased oxidative stress markers were observed. Tryptophan metabolism was upregulated, resulting in accumulation of tryptophan metabolites associated with reactive oxygen species generation, suggesting the possibility of oxidative stress as a mechanism of nongenotoxic carcinogenesis. D-Serine caused selective necrosis of the proximal straight tubules in the rat kidney accompanied by aminoaciduria, proteinuria, and glucosuria. LC-MS metabolomics was used to investigate urinary metabolic changes associated with D-serine-induced nephrotoxicity [82]. Increased serine, hydroxypyruvate, glycerate tryptophan, phenylalanine, and lactate and decreased methylsuccinic acid and sebacic acid were observed. General aminoaciduria included proline, methionine, leucine, tyrosine, and valine as well as increased acetyl carnitine. Metabolomics has been applied to nephrotoxicity

of numerous compounds including nifedipine, D-mannitol, ochratoxin A, sodium diclofenac, perfluorododecanoic acid, and thioacetamide [83–86].

5. METABOLOMICS IN CLINICAL NEPHROTOXICITY

5.1. Immunosuppressive drugs-induced nephrotoxicity

Mechanisms inherent to immunosuppressive nephrotoxicity have been discussed. Nephrotoxicity associated with CsA, sirolimus, and tacrolimus remains a major concern in hemodialysis and organ transplantation. Hemodialysis is an important alternative for renal replacement therapy. ^{1}H NMR metabolomics was used to study 57 kidney transplant recipients with normal allograft function and identify time-dependent changes in serum metabolites in response to CsA or tacrolimus immunosuppression [87]. Although lipid metabolites were increased in both, the CsA-treated group was higher. Metabolites included glucose, hypoxanthine, lactate, succinate, and taurine. These changed in response to treatment duration. In contrast, TMAO, known to be associated with graft dysfunction, did not differ.

Although CsA is effective against rejection, its use is limited by its nephrotoxicity. ^{1}H NMR and LC-MS metabolomics detected a number of metabolite changes due to CsA [88]. The presence of increased urinary 15-F_{2t}-isoprostane indicated oxidative stress. 15-F_{2t}-isoprostane was, on average, higher after CsA treatment versus placebo. While there were no conclusive changes in plasma 15-F_{2t}-isoprostane or metabolite patterns, nontargeted metabolomic analysis revealed significant changes in urine metabolites associated with negative effects on proximal tubule cells. The major urinary metabolites were citrate, hippurate, lactate, TMAO, creatinine, and phenylalanine. Previous studies showed that CsA caused urine metabolite pattern changes for proximal tubule damage and that these changes were more sensitive than traditional markers such as BUN and Scr [33]. Changes in urine metabolite patterns were sufficiently sensitive to detect the negative effects of CsA on the kidney.

5.2. Melamine

UPLC-MS has been employed to investigate urinary metabolites from melamine-exposed children. Most metabolites were related to the TCA cycle. Citric acid metabolism was disturbed by the effect of melamine on renal function. As such, this approach can provide a noninvasive examination of the melamine-induced renal injury [89].

5.3. Cancer

About 18,000–20,000 new cases of renal cancer are diagnosed each year in the United States. A substantial number of these cases have been associated with analgesic preparations containing phenacetin and exposure to lead and arsenic exposure. Renal cell carcinoma (RCC) is the sixth leading cause of cancer death and is responsible for 11,000 deaths per year in the United States. Approximately, one-third of patients are present with metastatic disease for which there is no adequate treatment currently. No biofluid screening tests exist for RCC.

Metabolomics was used to investigate RCC in humans [90]. RCC serum had increased lipid, isoleucine, leucine, lactate, alanine, *N*-acetylglycoproteins, pyruvate, and glycerol, with decreased acetoacetate, glutamine, phosphatidylcholine/choline, TMAO, and glucose. Altered metabolites were most likely the result of compensation via glycolysis to maintain energy homeostasis due to impaired TCA cycle. RCC tissues were characterized by increased lipid content [91]. Another study showed that increased urinary sorbitol was related to glycolysis [92]. A metabolomic platform including hydrophilic interaction chromatography, UPLC-MS, and GC-MS has been applied to detection of urinary RCC markers [93].

5.4. ARI

Low sensitivity of current biomarkers (BUN and Scr) limits their utility in early ARI. LC-MS serum metabolomics was applied to discovery of novel biomarkers in ARI [94]. Increased acylcarnitines, five amino acids, reduced serum arginine, and several lysoPCs were observed in these patients. Increased homocysteine, asymmetric dimethylarginine, and pyroglutamate have been recognized as ARI markers and acylcarnitines represent markers of defective fatty acid oxidation. Another LC-MS metabolomic analysis identified HVA-SO_4 as a biomarker in urine from ARI children [95]. Matrix-assisted laser desorption/ionization Fourier transform mass spectrometry has been used to analyze small molecule metabolites in urine from renal transplant recipients with varying levels of acute tubular injury [96].

6. CONCLUSION AND FUTURE PERSPECTIVES

^{1}H NMR or MS metabolomics is a powerful and reliable analytical approach for nephrotoxicity studies. NMR has been the primary technique due to its ease of use, inherent quantitative ability, and high reproducibility.

More recently, GC-MS or LC-MS has become more widely used due to its greater sensitivity and specificity. Continued advances in NMR spectroscopic hardware and increased parallel application of analytical platforms such as UPLC coupled with quadrupole time-of-flight mass spectrometry (UPLC-QTOF/MS) will enable even wider coverage of the metabolome. In addition, the associated statistical modeling of the complex spectral datasets is well established and biomarker information recovery continues to advance as novel tools are continually developed. The continued integration and parallel application of metabolomics with genomics, transcriptomics, and proteomics holds considerable promise to significantly extend our mechanistic understanding of complex nephrotoxicity. The analytical platforms and statistical approaches presented for this exemplar model toxin are equally applicable to the study of any given toxin or pharmaceutical in the preclinical and clinical setting. Hence, this "top-down" systems level approach may prove of immense utility in furthering our understanding of nephrotoxicity.

In this review, the application of metabolomics was evaluated with respect to nephrotoxicity biomarkers. Identification of new novel and highly specific biomarkers of drug-induced renal toxicity could improve development of more biocompatible therapeutics. A number of markers have been identified and confirmed by multiple studies. These could provide important diagnostic and prognostic markers especially in early stage disease. Additional studies are clearly warranted to further investigate these promising findings as to the role of metabolomics, in general, and its relationship to nephrotoxicity specifically.

ACKNOWLEDGMENTS

This study was supported by Program for the New Century Excellent Talents in University (NCET-13-0954) and Changjiang Scholars and Innovative Research Team in University (IRT1174) from Ministry of Education of China, National Natural Science Foundation of China (J1210063, 81202909, 81274025, 81001622), China Postdoctoral Science Foundation (2012M521831), Key Program for International S&T Cooperation Projects of Shaanxi Province (2013KW31-01), and As a Major New Drug to Create a Major National Science and Technology Special (2011ZX09401-308-034, 2014ZX09304-307-02).

REFERENCES

[1] D. Choudhury, Z. Ahmed, Drug-associated renal dysfunction and injury, Nat. Clin. Pract. Nephrol. 2 (2006) 80–91.
[2] E.A. Hoste, J.A. Kellum, Acute kidney injury: epidemiology and diagnostic criteria, Curr. Opin. Crit. Care 12 (2006) 531–537.

[3] M.A. Perazella, Drug-induced nephropathy: an update, Expert Opin. Drug Saf. 4 (2005) 689–706.
[4] J.K. Nicholson, J. Connelly, J.C. Lindon, E. Holmes, Metabonomics: a platform for studying drug toxicity and gene function, Nat. Rev. Drug Discov. 1 (2002) 153–161.
[5] D.S. Wishart, Applications of metabolomics in drug discovery and development, Drugs R&D 9 (2008) 307–322.
[6] A. Roux, D. Lison, C. Junot, J.F. Heilier, Applications of liquid chromatography coupled to mass spectrometry-based metabolomics in clinical chemistry and toxicology: a review, Clin. Biochem. 44 (2011) 119–135.
[7] R.D. Beger, J. Sun, L.K. Schnackenberg, Metabolomics approaches for discovering biomarkers of drug-induced hepatotoxicity and nephrotoxicity, Toxicol. Appl. Pharmacol. 243 (2010) 154–166.
[8] A. Zhang, H. Sun, P. Wang, Y. Han, X. Wang, Metabonomics for discovering biomarkers of hepatotoxicity and nephrotoxicity, Pharmazie 67 (2012) 99–105.
[9] K.J. Boudonck, D.J. Rose, E.D. Karoly, et al., Metabolomics for early detection of drug-induced kidney injury: review of the current status, Bioanalysis 1 (2009) 1645–1663.
[10] D.S. Wishart, Metabolomics in monitoring kidney transplants, Curr. Opin. Nephrol. Hypertens. 15 (2006) 637–642.
[11] Y.Y. Zhao, Metabolomics in chronic kidney disease, Clin. Chim. Acta 422 (2013) 59–69.
[12] E.Y. Xu, A. Perlina, H. Vu, et al., Integrated pathway analysis of rat urine metabolic profiles and kidney transcriptomic profiles to elucidate the systems toxicology of model nephrotoxicants, Chem. Res. Toxicol. 21 (2008) 1548–1561.
[13] V. Fanos, R. Antonucci, M. Zaffanello, M. Mussap, Nenatal drug induced nephrotoxicity: old and next generation biomarkers for early detection and management of neonatal drug-induced nephrotoxicity, with special emphasis on uNGAL and on metabolomics, Curr. Med. Chem. 19 (2012) 4595–4605.
[14] K.J. Boudonck, M.W. Mitchell, L. Német, et al., Discovery of metabolomics biomarkers for early detection of nephrotoxicity, Toxicol. Pathol. 37 (2009) 280–292.
[15] D. Portilla, S. Li, K.K. Nagothu, et al., Metabolomic study of cisplatin-induced nephrotoxicity, Kidney Int. 69 (2006) 2194–2204.
[16] M.C. van de Poll, P.B. Soeters, N.E. Deutz, K.C. Fearon, C.H. Dejong, Renal metabolism of amino acids: its role in interorgan amino acid exchange, Am. J. Clin. Nutr. 79 (2004) 185–197.
[17] N. Seiler, F. Raul, Polyamines and apoptosis, J. Cell. Mol. Med. 9 (2005) 623–642.
[18] H.M. Wallace, K. Niiranen, Polyamine analogues—an update, Amino Acids 33 (2007) 261–265.
[19] M. Sieber, D. Hoffmann, M. Adler, et al., Comparative analysis of novel noninvasive renal biomarkers and metabonomic changes in a rat model of gentamicin nephrotoxicity, Toxicol. Sci. 109 (2009) 336–349.
[20] E.M. Lenz, J. Bright, R. Knight, et al., Metabonomics with ^{1}H NMR spectroscopy and liquid chromatography-mass spectrometry applied to the investigation of metabolic changes caused by gentamicin-induced nephrotoxicity in the rat, Biomarkers 10 (2005) 173–187.
[21] P. Espandiari, J. Zhang, B.A. Rosenzweig, et al., The utility of a rodent model in detecting pediatric drug-induced nephrotoxicity, Toxicol. Sci. 99 (2007) 637–648.
[22] X. Ma, J.R. Idle, K.W. Krausz, et al., Urinary metabolites and antioxidant products of exogenous melatonin in the mouse, J. Pineal Res. 40 (2006) 343–349.
[23] M. Nava, F. Romero, Y. Quiroz, et al., Melatonin attenuates acute renal failure and oxidative stress induced by mercuric chloride in rats, Am. J. Physiol. Renal Physiol. 279 (2000) F910–F918.

[24] G. Sener, A.O. Sehirli, H.Z. Altunbas, et al., Melatonin protects against gentamicin-induced nephrotoxicity in rats, J. Pineal Res. 32 (2002) 231–236.
[25] E.M. Lenz, J. Bright, R. Knight, I.D. Wilson, H. Major, A metabonomic investigation of the biochemical effects of mercuric chloride in the rat using ^{1}H NMR and HPLC-TOF/MS: time dependent changes in the urinary profile of endogenous metabolites as a result of nephrotoxicity, Analyst 129 (2004) 535–541.
[26] J.C. Sun, S. Bhattacharyya, L.K. Schnackenberg, et al., Discovery of early urinary biomarkers in preclinical study of gentamicin-induced kidney injury and recovery in rats, Metabolomics 8 (2012) 1181–1193.
[27] H. Lv, L. Liu, Y. Zhang, et al., Ingenuity pathways analysis of urine metabonomics phenotypes toxicity of gentamicin in multiple organs, Mol. Biosyst. 6 (2010) 2056–2067.
[28] M. Gnanasoundari, L. Pari, Impact of naringenin on oxytetracycline-mediated oxidative damage in kidney of rats, Ren. Fail. 28 (2006) 599–605.
[29] E.M. Lenz, J. Bright, R. Knight, I.D. Wilson, H. Major, Cyclosporin A-induced changes in endogenous metabolites in rat urine: a metabonomic investigation using high field ^{1}H NMR spectroscopy, HPLC-TOF/MS and chemometrics, J. Pharm. Biomed. Anal. 35 (2004) 599–608.
[30] S.Y. Saad, T.A. Najjar, Effects of STZ-induced diabetes and its treatment with vanadyl sulphate on cyclosporine A-induced nephrotoxicity in rats, Arch. Toxicol. 79 (2005) 493–499.
[31] S.S. Padi, K. Chopra, Salvage of cyclosporine A-induced oxidative stress and renal dysfunction by carvedilol, Nephron 92 (2002) 685–692.
[32] J. Klawitter, J. Klawitter, E. Kushner, et al., Association of immunosuppressant-induced protein changes in the rat kidney with changes in urine metabolite patterns: a proteometabonomic study, J. Proteome Res. 9 (2010) 865–875.
[33] J. Klawitter, J. Bendrick-Peart, B. Rudolph, et al., Urine metabolites reflect time-dependent effects of cyclosporine and sirolimus on rat kidney function, Chem. Res. Toxicol. 22 (2009) 118–128.
[34] T.A. Gonwa, D.E. Hricik, K. Brinker, J.M. Grinyo, F.P. Schena, Improved renal function in sirolimus-treated renal transplant patients after early cyclosporine elimination, Transplantation 74 (2002) 1560–1567.
[35] N.J. Serkova, U. Christians, Biomarkers for toxicodynamic monitoring of immunosuppressants: NMR-based quantitative metabonomics of the blood, Ther. Drug Monit. 27 (2005) 733–737.
[36] J. Klawitter, J. Klawitter, V. Schmitz, et al., Low-salt diet and cyclosporine nephrotoxicity: changes in kidney cell metabolism, J. Proteome Res. 11 (2012) 5135–5144.
[37] K.P. Gartland, F.W. Bonner, J.K. Nicholson, Investigations into the biochemical effects of region-specific nephrotoxins, Mol. Pharmacol. 35 (1989) 242–250.
[38] A. Wilmes, A. Limonciel, L. Aschauer, et al., Application of integrated transcriptomic, proteomic and metabolomic profiling for the delineation of mechanisms of drug induced cell stress, J. Proteomics 79 (2013) 180–194.
[39] S.Y. Han, K.C. Mun, H.J. Choi, et al., Effects of cyclosporine and tacrolimus on the oxidative stress in cultured mesangial cells, Transplant. Proc. 38 (2006) 2240–2241.
[40] C. Huang, H. Lei, X. Zhao, H. Tang, Y. Wang, Metabolic influence of acute cyadox exposure on Kunming mice, J. Proteome Res. 12 (2013) 537–545.
[41] E. Holmes, J.K. Nicholson, A.W. Nicholls, et al., The identification of novel biomarkers of renal toxicity using automatic data reduction techniques and PCA of proton NMR spectra of urine, Chemometr. Intell. Lab. Syst. 44 (1998) 245–255.
[42] J.C. Park, Y.S. Hong, Y.J. Kim, et al., A metabonomic study on the biochemical effects of doxorubicin in rats using ^{1}H NMR spectroscopy, J. Toxicol. Environ. Health A 72 (2009) 374–384.

[43] L. Zhao, H. Gao, F. Lian, et al., ^{1}H NMR-based metabonomic analysis of metabolic profiling in diabetic nephropathy rats induced by streptozotocin, Am. J. Physiol. Renal Physiol. 300 (2011) F947–F956.
[44] F. Li, A.D. Patterson, C.C. Höfer, et al., Comparative metabolism of cyclophosphamide and ifosfamide in the mouse using UPLC-ESI-QTOFMS-based metabolomics, Biochem. Pharmacol. 80 (2010) 1063–1074.
[45] H. Wen, H. Yang, M.J. Choi, et al., Identification of urinary biomarkers related to cisplatin-induced acute renal toxicity using NMR-based metabolomics, Biomol. Ther. 19 (2011) 38–44.
[46] J.Y. Bae, B.M. Lee, E.Y. Han, et al., Urinary biomarkers for cisplatin-induced nephrotoxicity using metabolomic approach in vivo, Toxicol. Lett. 205S (2011) S217.
[47] H.S. Kim, T.H. Kim, Y.J. Lee, M.Y. Ahn, H.S. Kim, Metabolomic profiling of cisplatin-induced nephrotoxicity in human normal kidney HK-2 cells, FASEB J. 25 (2011) 1087.12.
[48] P. Espandiari, B. Rosenzweig, J. Zhang, et al., Age-related differences in susceptibility to cisplatin-induced renal toxicity, J. Appl. Toxicol. 30 (2010) 172–182.
[49] N.A. Santos, C.S. Catão, N.M. Martins, et al., Cisplatin-induced nephrotoxicity is associated with oxidative stress, redox state unbalance, impairment of energetic metabolism and apoptosis in rat kidney mitochondria, Arch. Toxicol. 81 (2007) 495–504.
[50] S.Y. Saad, M.M. Arafah, T.A. Najjar, Effects of mycophenolate mofetil on cisplatin-induced renal dysfunction in rats, Cancer Chemother. Pharmacol. 59 (2007) 455–460.
[51] U. Mengs, C.D. Stotzem, Renal toxicity of aristolochic acid in rats as an example of nephrotoxicity testing in routine toxicology, Arch. Toxicol. 67 (1993) 307–311.
[52] Y. Ni, M. Su, Y. Qiu, et al., Metabolic profiling using combined GC-MS and LC-MS provides a systems understanding of aristolochic acid-induced nephrotoxicity in rat, FEBS Lett. 581 (2007) 707–711.
[53] M. Chen, M. Su, L. Zhao, et al., Metabonomic study of aristolochic acid-induced nephrotoxicity in rats, J. Proteome Res. 5 (2006) 995–1002.
[54] W. Chan, Z. Cai, Aristolochic acid induced changes in the metabolic profile of rat urine, J. Pharm. Biomed. Anal. 46 (2008) 757–762.
[55] W. Chan, K.C. Lee, N. Liu, et al., Liquid chromatography/mass spectrometry for metabonomics investigation of the biochemical effects induced by aristolochic acid in rats: the use of information-dependent acquisition for biomarker identification, Rapid Commun. Mass Spectrom. 22 (2008) 873–880.
[56] S. Lin, W. Chan, J. Li, Z. Cai, Liquid chromatography/mass spectrometry for investigating the biochemical effects induced by aristolochic acid in rats: the plasma metabolome, Rapid Commun. Mass Spectrom. 24 (2010) 1312–1318.
[57] X. Liu, Y. Xiao, H.C. Gao, J. Ren, D.H. Lin, Metabonomic study of aristolochic acid I-induced acute renal toxicity urine at female and male C57BL/6J mice based on ^{1}H NMR, Chem. J. Chin. Univ. 31 (2010) 927–932.
[58] Y. Lou, J. Li, Y. Lu, et al., Aristolochic acid-induced destruction of organic ion transporters and fatty acid metabolic disorder in the kidney of rats, Toxicol. Lett. 201 (2011) 72–79.
[59] X. Zhang, H. Wu, P. Liao, et al., NMR-based metabonomic study on the subacute toxicity of aristolochic acid in rats, Food Chem. Toxicol. 44 (2006) 1006–1014.
[60] D.M. Tsai, J.J. Kang, S.S. Lee, et al., Metabolomic analysis of complex Chinese remedies: examples of induced nephrotoxicity in the mouse from a series of remedies containing aristolochic acid, Evid. Based Complement Alternat. Med. 2013 (2013) 263757.
[61] C. Ma, K. Bi, D. Su, et al., Serum and kidney metabolic changes of rat nephrotoxicity induced by Morning Glory Seed, Food Chem. Toxicol. 48 (2010) 2988–2993.
[62] C. Ma, K. Bi, M. Zhang, et al., Metabonomic study of biochemical changes in the urine of Morning Glory Seed treated rat, J. Pharm. Biomed. Anal. 53 (2010) 559–566.

[63] C. Ma, K. Bi, M. Zhang, et al., Toxicology effects of Morning Glory Seed in rat: a metabonomic method for profiling of urine metabolic changes, J. Ethnopharmacol. 130 (2010) 134–142.
[64] Y. Yu, C. Ma, K. Bi, et al., A metabonomic analysis of urine from rats treated with rhizome alismatis using ultra-performance liquid chromatography/mass spectrometry, Rapid Commun. Mass Spectrom. 25 (2011) 2633–2640.
[65] L. Wei, P. Liao, H. Wu, et al., Toxicological effects of cinnabar in rats by NMR-based metabolic profiling of urine and serum, Toxicol. Appl. Pharmacol. 227 (2008) 417–429.
[66] L. Wei, P. Liao, H. Wu, et al., Metabolic profiling studies on the toxicological effects of realgar in rats by ^{1}H NMR spectroscopy, Toxicol. Appl. Pharmacol. 234 (2009) 314–325.
[67] H. Wang, J. Bai, G. Chen, et al., A metabolic profiling analysis of the acute hepatotoxicity and nephrotoxicity of Zhusha Anshen Wan compared with cinnabar in rats using ^{1}H NMR spectroscopy, J. Ethnopharmacol. 146 (2013) 572–580.
[68] J.G. Bundy, D.J. Spurgeon, C. Svendsen, et al., Environmental metabonomics: applying combination biomarker analysis in earthworms at a metal contaminated site, Ecotoxicology 13 (2004) 797–806.
[69] I.A. Al-Nasser, Cadmium hepatotoxicity and alterations of the mitochondrial function, J. Toxicol. Clin. Toxicol. 38 (2000) 407–413.
[70] K.B. Kim, S.Y. Um, M.W. Chung, et al., Toxicometabolomics approach to urinary biomarkers for mercuric chloride (HgCl2)-induced nephrotoxicity using proton nuclear magnetic resonance (^{1}H NMR) in rats, Toxicol. Appl. Pharmacol. 249 (2010) 114–126.
[71] Y. Wang, M.E. Bollard, J.K. Nicholson, E. Holmes, Exploration of the direct metabolic effects of mercury II chloride on the kidney of Sprague-Dawley rats using high-resolution magic angle spinning ^{1}H NMR spectroscopy of intact tissue and pattern recognition, J. Pharm. Biomed. Anal. 40 (2006) 375–381.
[72] J. Feng, H. Liu, K.K. Bhakoo, L. Lu, Z. Chen, A metabonomic analysis of organ specific response to USPIO administration, Biomaterials 32 (2011) 6558–6569.
[73] Q. Chen, R. Zhang, W.M. Li, et al., The protective effect of grape seed procyanidin extract against cadmium-induced renal oxidative damage in mice, Environ. Toxicol. Pharmacol. 36 (2013) 759–768.
[74] R. Lei, C. Wu, B. Yang, et al., Integrated metabolomic analysis of the nano-sized copper particle-induced hepatotoxicity and nephrotoxicity in rats, Toxicol. Appl. Pharmacol. 232 (2008) 292–301.
[75] G.S. Hwang, J.Y. Yang, H. Ryu do, T.H. Kwon, Metabolic profiling of kidney and urine in rats with lithium-induced nephrogenic diabetes insipidus by ^{1}H-NMR-based metabonomics, Am. J. Physiol. Renal Physiol. 298 (2010) F461–F470.
[76] G. Xie, X. Zheng, X. Qi, et al., Metabonomic evaluation of melamine-induced acute renal toxicity in rats, J. Proteome Res. 9 (2010) 125–133.
[77] X. Zheng, A. Zhao, G. Xie, et al., Melamine-induced renal toxicity is mediated by the gut microbiota, Sci. Transl. Med. 5 (2013) 172ra22.
[78] L.K. Schnackenberg, J. Sun, L.M. Pence, et al., Metabolomics evaluation of hydroxyproline as a potential marker of melamine and cyanuric acid nephrotoxicity in male and female Fischer F344 rats, Food Chem. Toxicol. 50 (2012) 3978–3983.
[79] T.H. Kim, M.Y. Ahn, H.J. Lim, et al., Evaluation of metabolomic profiling against renal toxicity in Sprague–Dawley rats treated with melamine and cyanuric acid, Arch. Toxicol. 86 (2012) 1885–1897.
[80] J.R. Zgoda-Pols, S. Chowdhury, M. Wirth, et al., Metabolomics analysis reveals elevation of 3-indoxyl sulfate in plasma and brain during chemically-induced acute kidney injury in mice, Toxicol. Appl. Pharmacol. 255 (2011) 48–56.

[81] T. Ohta, N. Masutomi, N. Tsutsui, et al., Untargeted metabolomic profiling as an evaluative tool of fenofibrate-induced toxicology in Fischer 344 male rats, Toxicol. Pathol. 37 (2009) 521–535.
[82] R.E. Williams, H. Major, E.A. Lock, E.M. Lenz, I.D. Wilson, D-serine-induced nephrotoxicity: a HPLC-TOF/MS-based metabonomics approach, Toxicology 207 (2005) 179–190.
[83] J.K. Ellis, T.J. Athersuch, R. Cavill, et al., Metabolic response to low-level toxicant exposure in a novel renal tubule epithelial cell system, Mol. Biosyst. 7 (2011) 247–257.
[84] M. Sieber, S. Wagner, E. Rached, et al., Metabonomic study of ochratoxin A toxicity in rats after repeated administration: phenotypic anchoring enhances the ability for biomarker discovery, Chem. Res. Toxicol. 22 (2009) 1221–1231.
[85] H. Zhang, L. Ding, X. Fang, et al., Biological responses to perfluorododecanoic acid exposure in rat kidneys as determined by integrated proteomic and metabonomic studies, PLoS One 6 (2011) e20862.
[86] N.J. Waters, C.J. Waterfield, R.D. Farrant, E. Holmes, J.K. Nicholson, Metabonomic deconvolution of embedded toxicity: application to thioacetamide hepato- and nephrotoxicity, Chem. Res. Toxicol. 18 (2005) 639–654.
[87] C.D. Kim, E.Y. Kim, H. Yoo, et al., Metabonomic analysis of serum metabolites in kidney transplant recipients with cyclosporine A- or tacrolimus-based immunosuppression, Transplantation 90 (2010) 748–756.
[88] J. Klawitter, M. Haschke, C. Kahle, et al., Toxicodynamic effects of ciclosporin are reflected by metabolite profiles in the urine of healthy individuals after a single dose, Br. J. Clin. Pharmacol. 70 (2010) 241–251.
[89] Y. Wang, Z.T. Jiang, Q.L. Liang, et al., Metabonomics research of the influence of melamine to the urine metabolism of the children based on UPLC/TOF-MS, Chem. J. Chin. Univ. 31 (2010) 57–60.
[90] H. Gao, B. Dong, X. Liu, et al., Metabonomic profiling of renal cell carcinoma: high-resolution proton nuclear magnetic resonance spectroscopy of human serum with multivariate data analysis, Anal. Chim. Acta 624 (2008) 269–277.
[91] M.R. Tosi, M.T. Rodriguez-Estrada, G. Lercker, et al., Magnetic resonance spectroscopy and chromatographic methods identify altered lipid composition in human renal neoplasms, Int. J. Mol. Med. 14 (2004) 93–100.
[92] B. Perroud, J. Lee, N. Valkova, et al., Pathway analysis of kidney cancer using proteomics and metabolic profiling, Mol. Cancer 5 (2006) 64.
[93] T. Kind, V. Tolstikov, O. Fiehn, R.H. Weiss, A comprehensive urinary metabolomic approach for identifying kidney cancer, Anal. Biochem. 363 (2007) 185–195.
[94] J. Sun, M. Shannon, Y. Ando, et al., Serum metabolomic profiles from patients with acute kidney injury: a pilot study, J. Chromatogr. B 893–894 (2012) 107–113.
[95] R.D. Beger, R.D. Holland, J. Sun, et al., Metabonomics of acute kidney injury in children after cardiac surgery, Pediatr. Nephrol. 23 (2008) 977–984.
[96] J. Wang, Y. Zhou, M. Xu, et al., Urinary metabolomics in monitoring acute tubular injury of renal allografts: a preliminary report, Transplant. Proc. 43 (2011) 3738–3742.

CHAPTER FOUR

Metabolic Syndrome in Pediatrics

Romy Kursawe[1], **Nicola Santoro**[1,2]
Department of Pediatrics, Yale University School of Medicine, New Haven, Connecticut, USA
[1]The authors conceived and wrote the chapter.
[2]Corresponding author: e-mail address: nicola.santoro@yale.edu

Contents

Advances in Clinical Chemistry, Volume 65
ISSN 0065-2423
http://dx.doi.org/10.1016/B978-0-12-800141-7.00004-8

Abstract

The ever growing prevalence of childhood obesity is being accompanied by an increase in the pediatric population of diseases once believed to be exclusive of the adulthood such as the metabolic syndrome (MS). The MS has been defined as the link between insulin resistance, hypertension, dyslipidemia, impaired glucose tolerance, and other metabolic abnormalities associated with an increased risk of atherosclerotic cardiovascular diseases in adults. In this review, we will discuss the peculiar aspects of the pediatric MS and the role of novel molecules and biomarkers in its pathogenesis.

1. INTRODUCTION

The prevalence of childhood obesity has been increasing in the last four decades [1]. In 2010, it has been estimated that 43 millions of children were overweight worldwide and this number is expected to increase up to 60 million by 2020 [1]. The worldwide epidemic of childhood obesity is responsible for the occurrence in pediatrics of disorders once mainly found in adults, such as the metabolic syndrome (MS). Described by Gerald Reaven, the MS has been defined as "a link between insulin resistance, hypertension, dyslipidemia, impaired glucose tolerance and other metabolic abnormalities associated with an increased risk of atherosclerotic cardiovascular diseases in adults" [2]. While in adults the presence of the syndrome and the validity of its definitions are based on strong markers of cardiovascular diseases and can be used to predict the occurrence of cardiovascular complications, in childhood such outcomes are rare. This makes the several definitions proposed for the syndrome in pediatrics difficult to test and to validate. Also in childhood, the major cause of peripheral insulin resistance is obesity, in addition to a genetic background, low physical activity, specific dietary habits, and the ethnic background. Interestingly, obesity is not synonymous of insulin resistance as some obese adolescents may be very insulin sensitive and show a healthy metabolic phenotype [3]. Despite those differences, the core components for its definition in pediatrics are the same as in adults: a marker of adiposity (body mass index—BMI or waist circumference),

hypertension, altered glucose metabolism (impaired fasting glucose—IFG or IGT), elevated triglycerides (TGs), and low HDL cholesterol. Also, difficulties in defining the MS in children and adolescents derive from the lack of normative values for some components of the MS such as HDL, TGs, and waist circumference. That is why the definition of MS in pediatrics is driven by that given in adults.

The key factor in the pathogenesis of MS is insulin resistance, a phenomenon occurring mainly in obese subjects [4]. The main pathogenic feature of insulin resistance is the lipid accumulation in organs and tissues that usually do not store lipids (ectopic fat accumulation) such as the liver, pancreas, and the skeletal muscle [4]. The lipids accumulation interferes with the normal insulin signaling cascade and makes the tissues resistant to the insulin action. Interestingly, the resistance to the insulin seems to be limited to the glucose metabolism. Therefore, under these conditions while hyperinsulinemia is not able to suppress hepatic glucose production, it turns the liver into a "fat-producing factory" with all of its negative downstream effects, including the genesis of hypertriglyceridemia [4]. Also, the adipose tissue insulin resistance causes an increase in lipolysis with a consequent increased hepatic TGs synthesis and secretion [4]. This causes a massive accumulation of lipids into the liver, which results in hepatic steatosis and higher TGs production. From a molecular point of view, the link between lipid accumulation and insulin resistance seems to be represented mostly by the excessive diacylglycerol (DAG) synthesis [5,6]. The increased amount of lipids in the hepatocytes (but also in the skeletal muscle) has been shown to increase the amount of DAG, a signaling intermediate that activate members of the protein kinase C family, thus altering insulin signaling [6]. As a consequence of insulin resistance, the pancreas needs to increase its insulin production to maintain normal value of glycemia, promoting the lipid accumulation, further worsening insulin resistance, and generating a vicious cycle.

2. CLINICAL FEATURES OF CHILDREN WITH METABOLIC SYNDROME

All the metabolic changes driven by insulin resistance translate in dysglycemia and dyslipidemia in an obesity context. In spite of the emerging difficulties in transposing the definition of MS from adults to children, the MS in children is commonly defined as the co-occurrence of three or more of the following features: severe obesity (usually with a waist circumference higher than the 90th sex- and age-specific percentile), dyslipidemia

(increase of TGs and decrease of HDL), hypertension, and alterations of glucose metabolism such as IGT and type 2 diabetes (T2D).

Previously, the international diabetes federation consensus group proposed a definition to be used in the clinical setting [7]. The effort to reach an agreement on the definition of MS in pediatrics was made in order to overcome conflicts arising from different definitions. In fact, the numerous definitions used not only make it difficult to diagnose but also to follow the epidemiology of the MS in pediatrics. A study by Goodman *et al.* has clearly shown how changes in MS definition dramatically influence prevalence differences ranging from 15% to 50% according to definition used [8]. Although there is not complete agreement on the fine definition of MS in youth, given to the limitations mentioned above, the cornerstones of its definition still remain and need to be identified by the pediatricians.

2.1. Clinical measures of adiposity: BMI or waist circumference?

The BMI is a predictor of coronary artery disease (CAD) risk factors among children and adolescents [9,10] and its utility has been endorsed by International Obesity Task Force and Center for Disease Control (CDC) [11–13]. The cut-off points of CDC identify children with a BMI higher than 85th percentile as overweight and children with a BMI higher than 95th percentile as obese. Data from multiracial cohorts of obese children, in which obesity was defined according to the CDC cut-off, showed that the severity of obesity and the prevalence of MS are strongly associated [4]. However, obesity *per se* is not a marker sufficient for identifying children at risk for MS. The fat distribution plays an important role in influencing the occurrence of metabolic complications consequent to obesity. Visceral fat accumulation, in fact, is strongly associated to MS in childhood [14] and CAD later in the life [15] and is the best clinical predictor of visceral fat accumulation [16]. Although reference values for waist circumference in children do exist for Canada [17], Italy [18], the UK [19], and the United States [14] and cut-off points beyond which there is an increase of the prevalence of CAD risk factors have been provided [14], this measure is not commonly used in children, probably because no organization has endorsed waist circumference cut-off for children. The importance of measuring waist circumference is corroborated by studies in children and adolescents showing that subjects with high waist circumference values are more likely to have elevated CAD risk factors, compared with those with low waist circumference, within a given BMI category [15]; this means that waist circumference

may be, for such an extent, considered a more reliable measure for predicting MS than BMI alone. In fact, as in adults [20–23], in children an increased waist circumference has been correlated with abnormal systolic and diastolic blood pressure and elevated levels of serum cholesterol, LDL, TGs, insulin as well as with lower HDL concentrations [24–26]. The association between the clustering of cardiovascular risk factors and waist circumference is not only a reflection of the obesity degree, but it has a pathophysiological background, given that visceral adiposity is one of the main risk factors for the development of insulin resistance, diabetes mellitus, hypertension, and cardiovascular disease [27]. The mechanisms involved in these common clinical associations are not completely known, but include the impaired suppression of hepatic glucose production [28], the increased portal release of free fatty acids (FFAs), the increased visceral production of glycerol [29], and the abnormal production of adipose tissue-derived hormones and cytokines, such as tumor necrosis factor-alpha (TNF-α), leptin, and adiponectin [30,31]. The lack of normative data for the waist circumference in pediatrics has led many investigators to use corrected measures of the waist circumference, such as the waist-to-height ratio [32]. The waist-to-height ratio takes into account the body proportions and is maybe more precise in pediatrics than the simple measure of the waist circumference. Also, very recently, Groves *et al.* have shown that the prospective association between WHtR in childhood and cardiometabolic outcomes in adolescent boys [33]. In particular, the authors by using a secondary data analysis of the Avon Longitudinal Study of Parents and Children in a population of 2858 adolescents showed that adolescents who had a WHtR ≥ 0.5, the odds ratio of having three or more cardiometabolic risk factors was 6.8 [4.4–10.6] for males and 3.8 [2.3–6.3] for females [33].

2.2. Family history and acanthosis nigricans

The family history needs to be investigated, given that the heritability for each component of the syndrome has been well demonstrated [34,35]. In fact, heritability for obesity ranges from 60% to 80%, heritability for blood pressure varies from 11% to 37%, while for lipid levels it varies from 43% to 54% [34]. Moreover, a study by Weiss *et al.* has shown that those children who do not show MS early in childhood are less prone to develop it later, further supporting, indirectly, a strong genetic component in the development of MS [36].

Along with the family history, signs for insulin resistance such as acanthosis nigricans (AN must be checked carefully as it is a robust marker

of insulin resistance. Recently, Rafalson *et al.* described that the prevalence of AN quantifies its association with dysglycemia in an ethnically diverse group of eighth-grade students [37]. The authors showed that AN was in 28.2% of students and that the presence of AN was associated with a 59% increased likelihood of high-risk hemoglobin A1c (HbA1c): ($P=0.04$), twice the likelihood of IGT ($P=0.06$), and 47% greater likelihood of IGT/IFG combined ($P<0.0001$) [37]. Also the validity of each of those clinical features (adiposity, family history, and AN) has been recently demonstrated [38]. In fact, it has been recently observed that obese children with a positive family history for diabetes, AN, and a high waist-to-height ratio have almost four times higher odds to show the features of the MS than subjects without these clinical signs [38].

3. METABOLIC CHARACTERISTICS OF CHILDREN AND ADOLESCENTS WITH METABOLIC SYNDROME

Since insulin resistance represents the key pathogenic determinant in the development of the MS, all patients should be investigated for it. Weiss *et al.* have well demonstrated how the increase of insulin resistance parallels the increase of the risk of MS in obese children and adolescents [4]. Some studies have suggested a direct effect of hyperinsulinemia consequent to insulin resistance on each single component of the syndrome.

3.1. Hypertension

It has been shown that insulin resistance *per se* may cause hypertension. Insulin levels in children between 6 and 9 years seem to predict changes in blood pressure in adolescence [39], also the Bogalusa Heart Study has demonstrated a strong correlation between the persistent high-insulin levels and the development of CAD in children and young adults [40]. Some studies in children have also demonstrated that fasting insulin predicted the levels of blood pressure 6 years later [39]. While cross-sectional and longitudinal studies suggest a causal effect of insulin on the development of hypertension, the mechanisms underlying this relationship are not clear yet. As it has been suggested, in fact, a direct effect of hyperinsulinemia on hypertension may be ascribed to the effect of insulin on (i) sympathetic nervous system activity [41], (ii) sodium retention by kidney [42], and (iii) vascular smooth muscle growth stimulation [43].

3.2. Dyslipidemia

While the association between the insulin and the development of hypertension is not easy to explain from pathophysiological point of view, the effect of hyperinsulinemia on lipid metabolism is clearer. Several studies showed, in fact, that hyperinsulinemia stimulates the synthesis of TGs by increasing the transcription of genes for lipogenic enzymes in liver [44]. In an insulin-resistant environment, there is not only an increase of FFAs flux from the adipose tissue to the liver, where the FFAs serve as substrate for the TGs, but also an increase of *de novo* lipogenesis (DNL), due to the abundant availability of substrate and the upregulation of key lipogenic genes. In general, the synthesis of TGs in the liver is nutritionally regulated, and its formation from simple carbohydrates requires multiple metabolic pathways, including glycolysis and pyruvate oxidation to generate acetyl-CoA for fatty acid synthesis, NADPH generation to supply the reductive power, packaging of fatty acids into a glycerophosphate backbone, and finally, lipoprotein packaging to export TGs. Under fasting conditions, the contribution of *DNL* to TGs is small in humans [45]. It has been shown in obese adults that hepatic *DNL* contributes to hepatic TGs accumulation, which represents the hallmark of hepatic steatosis and to an elevation of fasting VLDL [45]. Moreover, recent data in lean insulin-resistant young subjects indicate that they have a marked defect in muscle glycogen synthesis and divert a lot of their ingested energy into *DNL*, resulting in increased hepatic TGs synthesis, increased plasma TGs and lower HDL [46]. These results suggest that insulin resistance, a condition commonly occurring in obese subjects, might contribute to the development of dyslipidemia and, eventually to the development of hepatic steatosis, by increasing hepatic *DNL*.

3.3. Puberty as cause of transient insulin resistance

Although obesity is the most important cause of insulin resistance among obese and adolescents, it is important to note that a transient insulin-resistant state occurs during puberty [47], and that this state worsens the insulin resistance present in obese children accelerating the progression to MS and T2D. In particular, Amiel *et al.* in 1984 firstly suggested that there is a certain and transient degree of impairment in insulin sensitivity during the puberty and that insulin resistance peaks in mid-puberty and reverses at the end of the adolescence [47]. Those changes have been ascribed to rise in growth hormone and IGF-1 during this period [48]. In healthy individuals, this phenomenon is balanced by an increased insulin secretion by the beta cell, but in

obese individuals, the co-occurrence of obesity and puberty represents the perfect storm causing such a high degree of insulin resistance that the beta cell is not always able to produce enough insulin to maintain the glycemic control.

3.4. Subtle inflammation in obese insulin-resistant youth

Along with insulin resistance, the MS is associated with a proinflammatory state [49], which in turn, is associated with a high CAD risk. It has been proven that obese children show an elevation of the C-reactive protein (CRP), which is a biomarker of inflammation and is also associated with adverse cardiovascular outcomes [4], that is why some authors suggested that an underlying inflammation may be an additional factor contributing to the long-term adverse cardiovascular outcomes independent of the insulin resistance degree [4].

4. PREDIABETES AND TYPE 2 DIABETES IN YOUTH

Another component closely related to insulin resistance, which is actually its consequence is the alteration of glucose metabolism, characterized by several stages (prediabetes) preceding the overt diabetes. According to American Diabetes Association (ADA) criteria, T2D is defined as fasting plasma glucose levels higher than 125 mg/dl or plasma glucose levels higher than 200 mg/dl 2 h after an oral glucose tolerance test (OGTT), while IGT is defined when plasma glucose levels are higher than 140 mg/dl 2 h after OGTT [50]. Along with IGT another prediabetic state has been individuated: IFG. IFG is defined as serum fasting glucose levels between 100 and 125 mg/dl [50]. Epidemiological studies indicate that IFG and IGT are two distinct categories of individuals and only a small number of subjects meet both criteria, showing that these categories overlap only to a very limited extent in children [51]. Recently, the ADA has published the recommendations to use HbA1c to diagnose diabetes [50]. In particular, it has been suggested a cut-off point of 6.5% to make diagnosis of diabetes. This cut-off point was chosen on the basis of cross-sectional and longitudinal studies conducted in adult subjects showing that a cut-off point of 6.5% identifies about one-third of cases of undiagnosed diabetes and that subjects with HbA1c higher than that cut-off have a long term higher prevalence of microvascular complications [3,52–54]. Subjects with HbA1c between 5.7% and 6.4% have been defined as "at increased risk of diabetes" [50]. It has to be noted that these two methods of diagnosis of T2D are not mutually exclusive, since

there is a little agreement between them [55]. Thus, for a correct diagnosis, it is useful to measure them both. The relationship between insulin demand and secretion is a key factor regulating the maintenance of normal glucose tolerance (NGT). In fact, the beta cell responds to insulin resistance occurring in obese children and adolescents by producing a state of hyperinsulinemia, which maintains normal glucose levels [56]. In the long run, however, the beta-cell function tends to deteriorate in some, and the insulin secretion may not be sufficient to maintain glucose levels within the normal range [56,57] (Fig. 4.1).

In fact, when insulin secretion is estimated in the context of the "resistant milieu," IGT subjects show a significantly lower degree of insulin secretion than the group with a NGT [3,58]. In particular, Weiss *et al.* investigated the role of insulin secretion in glucose regulation in a group of 62 obese adolescents with different glucose tolerance status (30 NGT, 22 with IGT and 10 with T2D) using hyperglycemic clamp studies [58]. This study showed that compared to obese adolescents with similar insulin resistance, those with IGT show a progressive loss of glucose sensitivity of beta-cell first-phase secretion and that the beta-cell second-phase secretion is compromised in T2D [51,58]. This does not mean that the defect of the first phase is less influential than the defect of the second phase in causing hyperglycemia,

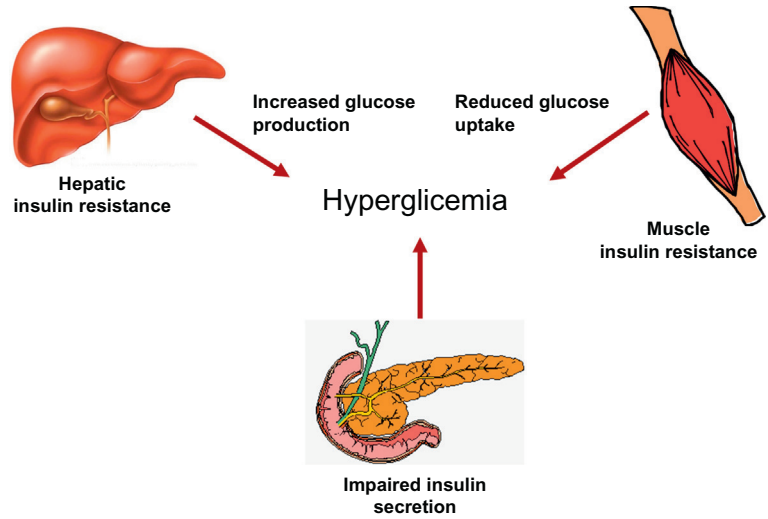

Figure 4.1 Physiopathology of hyperglycemia in prediabetes and type 2 diabetes. The figure shows the major events contributing to prediabetes and type 2 diabetes. In particular, the prediabetes and the overt diabetes develop only when the beta-cell insulin secretion is incapable to face the liver and muscle insulin resistance.

yet simply recognizes that the decline of the first phase of the insulin secretion is present before the overt diabetes and it may be considered the fingerprint of prediabetes, whereas the defect in the second phase is required for the development of T2D. In fact, differences in beta-cell function have been described in various prediabetic conditions seen in obese adolescents, such as IFG or IGT, or the combined IFG/IGT states. Cali *et al.* documented that in obese adolescents (i) IFG is primarily linked to alterations in glucose sensitivity of first-phase insulin secretion [51], (ii) IGT is characterized by a more severe degree of peripheral insulin resistance and reduction in first-phase secretion, and that (iii) the co-occurrence of IFG and IGT is the result of a defect in second-phase insulin secretion and a profound insulin resistance [51]. Surely, a genetic predisposition plays an important role in the development of T2D. This idea is supported by clinical studies showing that adolescents developing IGT or T2D show a lower insulin secretion even before the onset of IGT or T2D. The role of a "preexisting" beta-cell dysfunction in obese adolescents with NGT has been shown by Cali *et al.* in a longitudinal study [59]. In a group of obese NGT adolescents who underwent repeated OGTT over a period of 3 years, those who progressed to IGT had a lower beta-cell function at baseline compared to those who did not progress [59]. It is worth mentioning that changes in insulin secretion and sensitivity in adolescents occur quicker than in adults; in fact, while in adults the transition toward T2D takes about 10 years with about 7% per year reduction in beta-cell function [60,61], in obese adolescents it has been estimated that beta-cell function reduction occurs at a rate of about 15% per year [62], with a mean transition time from prediabetes to overt diabetes of about 2.5 years [57]. This means that T2D in adolescents might be a more severe disease than in adults and also that the genetic components may play a bigger role in pediatrics than in adults in the development of T2D [63].

5. NONALCOHOLIC FATTY LIVER DISEASE IN YOUTH

5.1. Association between fatty liver and metabolic syndrome

Nonalcoholic Fatty Liver Disease (NAFLD) has become the most common cause of liver disease in pediatrics [64]. It is defined by the presence of microvesicular steatosis in more than 5% of the hepatocytes in the absence of use medications, alcohol abuse, and other determinants that may result in fatty liver [65]. NAFLD encompasses a range of disease severity spanning from the simple steatosis to nonalcoholic steatohepatitis (NASH), which in turn can

progress to cirrhosis [65]. In an article using 742 autopsy specimens from children in San Diego County, 13% of subjects were found to have NAFLD, with the highest rate found in Hispanics (11.8%), and the lowest in African Americans (1.5%) [64]. The natural history of NAFLD is not entirely known; nevertheless, mortality among NASH patients is higher than in those with NAFLD without fibrosis or inflammation [66]. Alarming data have been shown in pediatric population. NASH is increasingly recognized in obese children and it has been demonstrated that it may progress to cirrhosis in this age group [66]. Also, the metabolic complications of NAFLD have been well defined. In fact, recent studies in obese adolescents demonstrated that increased ALT levels are associated with deterioration in insulin sensitivity and glucose tolerance, as well as with increasing FFA and TG levels [67]. Also, the relationship between fatty liver and glucose dysregulation has been demonstrated in a multiethnic group of 118 obese adolescents [68]. The cohort was stratified according to tertiles of hepatic fat content, measured by fat gradient MRI. All children underwent an oral glucose tolerance test and insulin sensitivity was estimated by the Matsuda index and HOMA-IR. Independently of obesity, the severity of fatty liver was associated with the presence of prediabetes (IGT and IFG/IGT) [68]. In fact, paralleling the severity of hepatic steatosis, there was a significant decrease in insulin sensitivity and impairment in beta-cell function as indicated by the fall in the disposition index [68]. Further studies showed that the pronounced dyslipidemic profile associated with fatty liver in pediatrics is characterized by large VLDL, small dense LDL, and decreased large HDL concentrations [69]. A study from D'Adamo *et al.* has highlighted the role of hepatic fat content in modulating insulin sensitivity [70]. The authors studied two groups of subjects with similar visceral fat and intramyocellular lipids: one group without and the other one with hepatic steatosis and showed that obese individuals with steatosis had increased muscular and hepatic insulin resistance [70]. More recently, in a longitudinal study, we observed that baseline hepatic fat content correlates with 2 h glucose and insulin sensitivity and secretion at follow-up [71]. These data clearly indicate that the deleterious effect of intrahepatic fat accumulation influences the insulin sensitivity at a multiorgan level playing a bigger role than the other ectopic compartments [71]. In general, obese children and adolescents with hepatic steatosis tend to show an adverse metabolic pattern as characterized by dyslipidemia and adverse changes in glucose metabolism. We have recently shown that liver damage correlates with insulin resistance in obese children [72]. In particular, the levels of the caspase-cleaved CK-18

fragment (CK-18), a robust biomarker of liver damage, are inversely correlated with insulin sensitivity, meaning that not only the amount of intrahepatic fat but also the degree of steatohepatitis may affect insulin sensitivity [72]. Interestingly, this association is present in Caucasian and Hispanic obese children and adolescents, but not in African Americans. In the latter population, there seems to be dissociation between the degree of liver injury and insulin sensitivity [72]. This data is consistent with the data shown by Guerrero *et al.*, showing a clear dissociation between the amount of liver fat and the degree of insulin sensitivity in African Americans [73]. The cause of this difference among ethnic groups is not known, but it is likely that the genetic background and the interaction between gene variants and nutrients may be a major determinant of such differences.

5.2. Pathogenesis and progression of NAFLD

The most credited model for the pathogenesis of NAFLD is the "two-hit" theory, where the first hit is represented by the insulin resistance, responsible for the abnormalities in lipid storage and lipolysis therefore leading to an increased fatty acids flux from adipose tissue to the liver and to subsequent accumulation of TGs into the hepatocytes [45]. While, the "second hit" might be represented by the oxidative stress, which activates the inflammatory cascade and generates reactive oxygen species (ROS) such as hydroxyl radicals and superoxide anions, which react with the excess lipid to form peroxides [74]. The synthesis of TGs in the liver is nutritionally regulated and has two main routes: adipose tissue lipolysis and hepatic DNL, which account, respectively, for about 60% and 25% of hepatic fat accumulation, only a little fraction of the liver fat comes directly from the chylomicron remnants [45]. In fact, insulin-resistant individuals have a reduced ability to suppress FFAs flux from the adipose tissue. Therefore, the increased amount of FFA from adipose tissue lipolysis causes an increased formation of TGs in the liver. The formation of TGs from simple carbohydrates requires multiple metabolic pathways, including glycolysis and pyruvate oxidation to generate acetyl-CoA for fatty acid synthesis, NADPH generation to supply the reductive power, packaging of fatty acids into a glycerophosphate backbone, and finally, lipoprotein packaging to export TGs. When liver is saturated with glycogen (roughly 5% of liver mass), any additional glucose taken up by hepatocytes is shunted into pathways leading to the synthesis of fatty acids, which will be esterified in TGs and

exported to adipose tissue as VLDL. It has been shown in obese adults that hepatic *DNL* contributes to hepatic TGs accumulation, which represents the hallmark of hepatic steatosis, and to an elevation of fasting VLDL [45]. Once the ability of the liver to form TGs from FFA is saturated, the accumulation in the hepatocytes of FFA probably triggers the inflammation and oxidative pathways responsible for the progression of the disease from simple NAFLD to NASH. In fact, the products of peroxidation may injure cells directly by interfering with membrane function or stimulate fibrosis by hepatic stellate cells. In particular, oxidative stress is associated with an increased production of ROS and proinflammatory cytokines [75,76]. One of the effects of ROS is to cause lipid peroxidation of some lipids, such as the polyunsaturated fatty acids (PUFA) generating metabolites that are deleterious for the hepatocyte [75]. The lipid peroxidation leads to the activation of Kupffer cells with the production of inflammatory cytokines such as the TNF-α and to the activation of stellate cells, which in turn will favor neutrophils chemotaxis as well as liver fibrosis [74]. A large body of evidence suggests that the quality of dietary fat can influence the development of NAFLD [77,78]. In particular, recent literature provides clues that the dietary imbalance between omega-6 (*n*-6) and omega-3 (*n*-3) PUFA leads to development of NAFLD [79]. Individuals with NAFLD, in fact, have been shown to have a lower dietary intake of *n*-3 PUFA than healthy controls and an increase in the *n*-6/*n*-3 ratio consume in the diet [79,80]. Consistent with these data, lipidomic studies have shown that the intrahepatic fat in subjects with steatohepatitis is composed by an excess *n*-6 PUFA [81]. Also, we have recently shown that the excess of oxidized lipids derived from the linoleic acid (the precursor of the omega-6 PUFA) is associated with both liver injury and insulin secretion [82]. The pathogenetic mechanism linking these compounds to liver damage may be related to their effect on the PPAR-α, a potent modulator of lipid transport and oxidation. Also, the PUFA metabolites such as eicosanoids or oxidized fatty acids have one to two orders of magnitude greater affinity for PPAR-α and are consequently far more potent transcriptional activators of PPAR-α-dependent genes than their ancestry compounds [83]. Therefore, in a liver rich of *n*-6 PUFA, the continuous production of oxidized fatty acids perpetuates and enhances the intracellular oxidative stress leading to a sterile inflammation. Also, some data suggest that oxidized species deriving from the omega-6 PUFA may play a role in the impaired insulin secretion observed in subjects with fatty liver [82]. This phenomenon may be mainly due to the fact that the increase

omega-6 PUFA in the beta cell affects the glucose, amino acids, and GLP-1-stimulated insulin secretion and renders the beta cell strongly susceptible to cytokine-induced cell death.

5.3. Common gene variants associated with hepatic steatosis and liver damage

The field known as "genetics of NAFLD" is relatively new, since it started in 2008 with the discovery that the nonsynonymous SNP (rs738409) in the *PNPLA3* gene [84] is associated with fatty liver, high-ALT, and severity of liver disease [84,85]. This gene variant is characterized by a C to G substitution encoding an isoleucine to methionine substitution at the amino acid position 148 in the patatin-like phospholipase domain-containing protein 3 gene (*PNPLA3*). Subsequently, other studies confirmed this association, indicating an association also with the severity of NAFLD in adults as well as in children [86,87]. Moreover, it has been shown that this variant interacts with environmental stressors including obesity [88,89], carbohydrate intake [90], and alcohol consumption [91] that induce fatty liver. Therefore, these stressors seem to uncover the association between the rs738409 minor allele (G) and hepatic injury in populations in whom it is otherwise covert. The *PNPLA3* gene product, known as patatin-like phospholipase domain-containing protein 3 or adiponutrin, is a transmembrane protein localized in the membrane of lipid droplets that was originally identified as a member of the calcium-independent phospholipase A2 family [92]. However, it has both triacylglycerol hydrolase and acylglycerol transacetylase activity [92]. In animals and humans, adiponutrin is primarily expressed in white adipose tissue (WAT) and liver, its expression is nutritionally regulated, and it increases with obesity [92]. Recently, more insights concerning the PNPLA3 function have been provided. In particular, *in vitro* and animal studies have demonstrated that (i) PNPLA3 promotes TGs and phospholipid synthesis; (ii) PNPLA3 acts as a nutritionally regulated acyl-transferase, with an increased lipogenic activity under high-sucrose (glucose + fructose) diet; and (iii) the rs738409 causes a gain of the lipogenic function of the PNPLA3 [93]. Also, recently we showed an association between the rs2645424 in the *FDFT1* gene and CK-18, a robust marker of liver injury, in a multiethnic cohort of obese youths [72]. This variant, in fact, was found to be associated with NASH activity score in a cohort of adult women by a GWAS study performed in sample of 236 non-Hispanic white women with NAFLD [94]. The *FDFT1* gene, located on chromosome 8, is a key regulator of cholesterol biosynthesis [95,96], it

encodes the squalene synthase, an enzyme involved in sterol synthesis; in particular, it converts two molecules of farnesyl pyrophosphate into squalene, which is a precursor to cholesterol. Since the rs2645424 is an intronic variant, it is difficult to speculate on how it may affect the enzyme activity; it is possible that this SNP is in linkage disequilibrium with a variant in the promoter that enhancing its expression leads to an increased activation of the squalene synthase and to the intrahepatic accumulation of cholesterol. Animal studies have, in fact, shown that transient overexpression of the *FDFT1* gene in the liver of both wild-type and *LDLR* knockout mice resulted in increased *de novo* cholesterol biosynthesis, over-secretion of cholesterol-rich LDL, higher cholesterol levels, and a 37% increase in liver weight compared with controls due to hepatocyte proliferation [96]. This hypothesis would also be consistent with recent studies showing the role of intrahepatic cholesterol accumulation in the pathogenesis of NASH [97]. Another gene variant strongly associated with fatty liver in obese youth is the *GCKR* rs1260326. We observed that the minor (T) allele of the *GCKR* rs1260326 is associated with higher serum TGs, large VLDL levels, and small LDL size particles in obese children and adolescents [98]. Interestingly, we also found that the *GCKR* rs1260326 minor allele is associated with fatty liver [98]. This association is probably explained by the effect of the mutation on the protein. In fact, the rs1260326 is a functionally relevant SNP consisting of a C to T substitution coding for a proline to leucine substitution at the position 446 (P446L). A recent study has shown that the leucine in P446L confers to the protein a reduced capability to respond to fructose 6-phosphate, resulting indirectly in a constant increase in GCK activity in the liver [99], which leads to higher glycolytic flux and hence increasing the glucose uptake by the liver. The increased glycolysis would raise the levels of malonyl CoA, which in turn may favor the increase in TG levels through two different mechanisms either by serving as a substrate for DNL or by inhibiting carnitine-palmitoyl transferase-1, thus blocking fatty acid oxidation [99]. We have observed that the increase in large VLDL is actually consistent with this pathway. The increase of large VLDLs, which represent the youngest VLDL, may be probably due to both an increased synthesis of TGs into the liver as a consequence of an increased DNL, whose contribution to the accumulation of fat into the liver in obese adults is well established [45], and a reduced beta oxidation. Along with the increase of liver fat content, TGs and large VLDL, subjects carrying the T allele showed smaller LDL size, and slightly lower HDL cholesterol [98]. Thus, all together, subjects carrying the T allele seem to display a proatherogenic profile.

6. ASSOCIATION BETWEEN "LIPID PARTITIONING" AND METABOLIC SYNDROME

Along with intrahepatic fat accumulation, other compartments concur to the metabolic phenotype in obese youth. Interestingly, some severely obese adolescents may be very insulin sensitive and have a healthy metabolic phenotype [3]. Lipid partitioning pattern, rather than absolute degree of obesity, is the determinant of the metabolic effects of excess lipids. The term "lipid partitioning" refers to the distribution of body fat in organs (some of which are insulin-responsive tissues) and compartments. The majority of excess fat is stored in its conventional subcutaneous depot, yet other potential storage sites exist as well, such as the intra-abdominal (visceral) fat compartment and insulin-responsive tissues such as muscle, liver, the pancreas, and large blood vessels. The pattern of lipid storage not only has local, direct autocrine effects on signal transduction pathways but also determines the secretion profile of specific adipocytokines (described below) and inflammatory cytokines, thus affecting metabolism in distant organs via an endocrine effect [100,101]. The combined effect of these factors determines the sensitivity of insulin-mediated pathways within insulin-responsive organs (such as muscle and liver) and impacts the vascular system by affecting endothelial function. Taksali *et al.* described a potential nonfavorable pattern of lipid partitioning with excess intra-abdominal fat in relation to abdominal subcutaneous fat [102]. Obese children with a high visceral-to-subcutaneous fat ratio are not more obese (by BMI or by percent body fat) in comparison to those with a lower ratio, but have a greater degree of insulin resistance and an adverse metabolic phenotype manifesting as more severe dyslipidemia, systolic blood pressure, and dysglycemia [102,103]. A potential explanation for this phenomenon is that the intra-abdominal fat deposits drain directly into the liver where a postulated increase FFA flux accumulates. While intra-abdominal fat does have a greater sensitivity to adrenergic stimulation and a lower sensitivity to insulin *in vitro*, the contribution of this deposit to circulating FFAs concentration seems to be in proportion to its absolute quantity; therefore, its adverse effects may be related to other factors [104]. Differences in the abundance of lipid droplet proteins that regulate the storage and breakdown of TGs in the fat cell could play a key role. It has been suggested that when energy accumulates in adipocytes, the perilipin border of the fat vacuole breaks down, causing the adipocyte to demise [105]. Cell death then causes a recruitment of macrophages via tissue-derived

chemokines, especially in the visceral compartment. The accumulation of these macrophages results in increased secretion of inflammatory cytokines, initiating a proinflammatory milieu, which possibly drives the development of systemic insulin resistance, altered glucose metabolism, and endothelial dysfunction [105]. Obesity has been shown to be associated with subclinical inflammation in adolescents and more so in those who met the criteria of the MS.

Analyses of subcutaneous and visceral adipose tissue (VAT) depots from insulin-sensitive and resistant patients indicate that the insulin-sensitive and -resistant patients differ with respect to AMP-activated protein kinase (AMPK) activity and oxidative stress in all of their fat depots [106] and in the expression of genes related to inflammation, mitochondrial function, sirtuin 1 (SIRT1)/nicotinamide phosphoribosyltransferase (Nampt), and many others [106–109] in selected depots. Key abnormalities appear to be impaired TG storage and increased lipolysis by lipid droplets, mitochondrial dysfunction, inflammation, and increases in oxidative and endoplasmic reticulum stress [109,110]. Many of these abnormalities could be related to increased synthesis and release of chemokines from the adipocytes or more likely adjacent vascular cells that attract monocytes (CD68), T (CD4) and B lymphocytes, and neutrophils (MPO) from circulating blood [111]. The resultant increases in the release of FFA, ROS, and inflammatory cytokines and the decreased release of adiponectin from the adipocyte are thought to act on peripheral tissues to cause such disorders as T2D, atherosclerosis, and NAFLD. In subcutaneous abdominal fat, the indicated changes may also be associated with decreased capillarity [107] and impaired O_2 consumption and increased synthesis of type VI collagen [107,112], all of which could limit adipose tissue expansion.

7. IS THERE A HEALTHY METABOLIC PHENOTYPE IN OBESITY?

Several pathophysiological explanations for the MS have been proposed involving insulin resistance, chronic inflammation, and ectopic fat accumulation following adipose tissue saturation [107]. However, current concepts create several paradoxes, including limited cardiovascular risk reduction with intensive glucose control in diabetics [113], therapies resulting in weight gain (PPAR agonists) [114]. A newer prospective is the functional failure of the adipose tissue as an organ unable to buffer postprandial lipids [115].

The functions described for WAT from classical physiology are: heat insulation, mechanical cushioning, and storage site for fat in the form of TGs. However, this view has been dramatically changed with the recognition of the adipose tissue as a key endocrine organ. Adipose tissue secretes active endocrine, paracrine, and autocrine substances in response to different stimulus. Some of them are mainly released by the adipose tissue (i.e., leptin), while others are shared with other systems (i.e., TNF-α) thus interweaving its function in systemic whole-organism regulations. The current hypotheses consider that adipose tissue switches between two states: (i) avidly draining FFAs that come mainly from TG-rich lipoproteins during the postprandial period and (ii) gently releasing them during the fasting period [115]. Switching between one state and the other is most probably regulated by a multifactorial system including substrate and hormone levels, but also the functional state of the adipose tissue itself. A failure of the adipose tissue function in taking up dietary fat (being permanently switched to releasing FFAs) might lead to an excess of lipid flux toward other tissues, during the postprandial period and even during the fasting period, and to a decreased clearance of TG-rich lipoprotein particles [116]. The functional capacity of the adipose tissue varies among subjects explaining the incomplete overlapping among the MS and obesity and these differences may be explained by the different genetic background. In fact, variations at multiple gene loci seem to be partially responsible for these interindividual differences. Two candidate genes regulating lipid partitioning are the adiponectin (*APM1*) and the perilipin (*PLIN*) gene [117], which will be discussed in detail later.

Lipid partitioning has been proven to be a better determinant for insulin resistance then obesity alone. The distribution of body fat varies between men and women, men start to accumulate fat in the visceral depot far earlier than women, as well as between different ethnicities, with African Americans having increased subcutaneous fat storage capacity [118]. Therefore, a more healthy phenotype of obesity is an increased subcutaneous versus visceral abdominal fat ratio and a higher gluteal–femoral than abdominal fat distribution—the so-called pear shape. We showed in multiple studies that an unhealthy abdominal lipid partitioning with low subcutaneous and high visceral fat storage is associated with ectopic fat in muscle and liver, resulting in insulin resistance in youth [3,102,103].

8. ROLE OF SUBCUTANEOUS FAT IN THE PATHOGENESIS OF METABOLIC SYNDROME

Numerous investigators have shown increased circulating biomarkers of inflammation in MS, thus providing support for the syndrome's

proinflammatory state. However, there is a paucity of data on subcutaneous adipose tissue (SAT) biology in the pathogenesis of MS [119]. The subcutaneous fat—which comprises ~80% of adipose tissue—is readily accessible to study and has been shown to be metabolically correlated to indices of insulin resistance as well as to VAT [120–123]. In addition to intra-abdominal fat, investigators have shown that the amount of SAT positively correlates with increasing MS factor scores and negatively correlates with circulating adiponectin levels [124]. Other investigators have also reported that SAT is significantly associated with MS and increases with the increasing number of MS features, independent of age and sex [125]. Furthermore, inflammatory cells and processes, such as macrophage infiltration, appear to be important in adipose tissue inflammation. Specifically, investigators have examined abdominal SAT from obese subjects and reported that an inflamed adipose phenotype characterized by tissue macrophage accumulation in crown-like structures is associated with systemic hyperinsulinemia and insulin resistance and impaired endothelium-dependent flow-mediated vasodilation [126]. Macrophage retention in fat has been also linked to upregulated tissue CD68 and TNF-α mRNA expressions in addition to increased plasma high-sensitivity CRP concentrations.

9. NOVEL BIOMARKERS OF METABOLIC SYNDROME

As mentioned earlier—multiple clinical definitions have been suggested for diagnosing the MS in children and adolescents. It is of great interest and importance to identify biomarkers in patients prone to develop the syndrome during childhood (mainly those with significant obesity and/or a strong family history of CVD at young age or T2DM). Recently, several novel biomarkers have been identified and shown to be associated with early alterations of the metabolic milieu that characterize the initiation of the pathophysiological processes that culminate in accelerated atherogenesis and peripheral insulin resistance. While some of the biomarkers described herein may still be in their investigational phase and do not yet have any clinical implications, they shed light on the underlying pathophysiology of the MS and may serve to identify novel targets for early preventive interventions (Fig. 4.2).

9.1. The fibroblast growth factor 21

The search for metabolic markers able to identify subjects at high-metabolic risk and with a potential therapeutic impact has led to the discovery of the FGFs, several of which are implicated in glucose uptake and mitochondrial

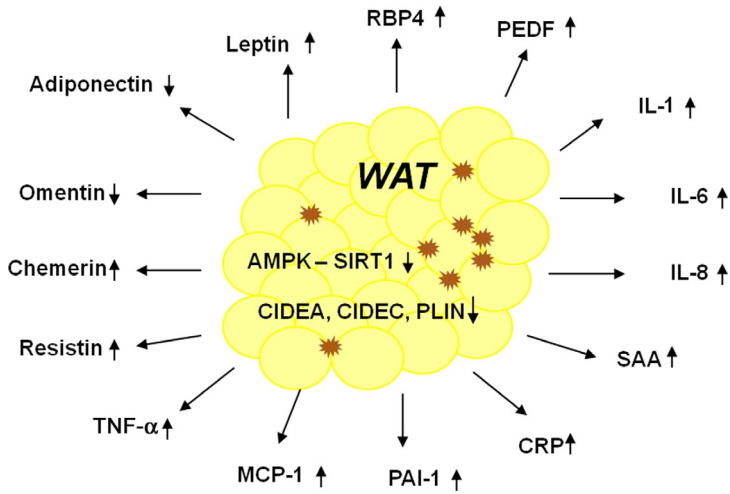

Figure 4.2 Adipokines involved in the pathophysiology of the Metabolic syndrome. The figure shows the most important adipokines associated with the Metabolic syndrome. Abbreviations: WAT, white adipose tissue; RBP, retinol-binding protein; PEDF, pigment epithelium-derived factor; IL, interleukin; TNF-α, tumor necrosis factor-alpha; MCP, monocyte chemoattractant protein; PAI, plasminogen activator inhibitor; CIDE, cell death-inducing DFF45-like effector; PLIN, perilipin; AMPK, AMP-activated protein kinase; SIRT, sirtuin.

function. Among them, fibroblast growth factor 21 (FGF-21) has become of particular interest. The FGF-21 is involved in the regulation of glucose, lipid, and energy metabolism *in vivo*. It acts through a receptor complex consisting of a coreceptor, β-klotho, and a FGF receptor, which is crucial for the FGF-21 specificity of the target cells [103,127] and elicits intracellular signaling cascades, including phosphorylation of FGF receptor substrate-2 and ERK [128,129]. Animal studies have shown that FGF-21 overexpression is protective against diet-induced obesity and insulin resistance [130], while the administration of FGF-21 causes a reduction of blood glucose, insulin, TG, cholesterol levels, steatosis, and obesity [130]. The role of FGF-21 in glucose and lipid metabolism is also inferred due to FGF-21 expression being highly regulated by feeding and, in particular, by dietary fat and carbohydrates [131–133]. More interestingly, the intracerebroventricular infusion of FGF-21 in high-fat diet-induced obese male rats increases hepatic insulin sensitivity, indicating that the CNS plays a pivotal role in the interplay between FGF-21 and glucose metabolism [130]. Despite its apparent antiobesity and antidiabetes effects, FGF-21 levels are elevated in obese and diabetic patients, suggesting a state of FGF-21 resistance analogous to

what is observed for leptin and insulin. Although, the entire picture explaining the role of FGF-21 in the pathogenesis of insulin resistance and the MS is still confused, some recent observations have suggested intriguing mechanisms possibly underlying this association. Animal studies have shown that FGF-21 expression in the liver is induced by fasting in wild-type mice but not PPAR-α knockout mice [134], thus suggesting that hepatic FGF-21 expression may be induced by the activation of PPAR-α. Moreover, when FFAs activate PPAR-α, the latter induces the expression of FGF-21 [134]. Furthermore, fasted FGF-21-knockout mice show increased lipolysis in adipocytes, which resulted in decreased adipocyte size and increased serum FFAs levels [134], indicating that FGF-21 could inhibit lipolysis in adipocytes during fasting. Although this mechanism may explain how FGF-21 can "protect" from ectopic fat deposition and the related insulin resistance, it is still hard to conceive how the FGF-21 resistance is established. More recently, studies in youth have shown that FGF-21 levels correlate not only with the obesity degree (being higher in obese subjects) but also with the presence of fatty liver [135].

9.2. Leptin

Leptin was the first identified adipocytokine, it is primarily expressed in adipose tissue and its serum concentration is directly proportional to body fat [136]. However, recent studies have confirmed that other tissues also express leptin, including placenta, ovaries, skeletal muscle, stomach, pituitary, and liver [137]. Leptin circulating levels are increased during a meal, whereas leptin levels decrease with fasting [138,139]. Leptin expression may also be increased by the actions of insulin, glucose, estrogens, glucocorticoids, TNF-α, and interleukin (IL-1), and in conditions of impaired renal function and acute inflammation [140]. A decrease in leptin levels is observed in response to beta-receptor agonists, androgens, cold exposure, thiazolidinedione, and cigarette smoking [141]. It is noted that the addition of leptin to cells in culture was found to promote proliferation and to inhibit apoptosis [142–144].

Leptin acts as an afferent satiety signal, regulating appetite, and weight in both humans and rodents. It affects central circuits in the hypothalamus, thereby suppressing food intake and stimulating energy expenditure. Thus, leptin plays a major role in the control of body fat stores through coordinated regulation of feeding behavior, metabolism, the autonomic nervous system, and body energy balance. Recent studies have revealed direct effects of

leptin in the periphery, partly through interactions with other peripherally acting hormones such as insulin [145]. Besides the neuroendocrine effects of leptin in the control of food intake and energy expenditure, binding of this hormone has been proven in lung, intestine, kidney, liver, skin, stomach, heart, spleen, and other organs [142,146], suggesting its pleiotropic actions. These include, for example, the role of leptin in the direct regulation of immune cells, pancreatic beta cells, adipocytes, muscle and blood cells [146]. Thus, leptin appears to act as an endocrine and paracrine factor for the regulation of puberty and reproduction; it affects maternal, placental, and fetal function; modifies insulin sensitivity in the muscle or liver; prevents ectopic lipid deposition; and links the endocrine and immune systems in the context of skin repair [145]. Many new peripheral actions of leptin have been demonstrating that leptin plays a role in modulating metabolism, energy expenditure, and pathological processes in humans [147,148].

9.3. Adiponectin

One of the most interesting biomarkers of MS remains adiponectin, which is secreted by the adipose tissue and abundant in plasma [149]. While other adipokines (resistin, visfatin, leptin, pigment epithelium-derived factor (PEDF), retinol-binding protein-4 (RBP4), and FGF-21) are increased in a dysmetabolic state, adiponectin is the only adipokine inversely correlated with insulin sensitivity and that is low in subjects with MS or T2D [149]. Several lines of evidence, consisting of clinical association studies in humans, genetic studies, experimental animal studies and models, and *in vitro* studies support the protective role of adiponectin in the development of obesity-related disorders and the MS, particularly in the pathogenesis of T2D, and cardiovascular disease. Human studies have clearly shown an association between adiponectin levels and the MS, as well as T2D. In particular, dysregulation of the expression and secretion of adiponectin may play a role in the pathogenesis of T2D [4,150–152]. In fact, it has been shown that low adiponectin levels are an independent risk factor for the progression of T2D [153–155].

Although adiponectin has been shown to be associated with insulin resistance in a large number of studies, its effect is not limited to glucose metabolism, since adiponectin has also been shown to play a role in lipid metabolism [153–155] and inflammation [156]. Some animal and *in vitro* studies have studied the biological pathway by which adiponectin modulates insulin sensitivity. In fact, in models of genetic- and diet-induced obesity,

and *in vitro* experiments have shown that adiponectin causes improvements in insulin sensitivity in muscle cells [156–158] and stimulates fatty acid oxidation and glucose uptake in skeletal muscle [158,159] and adipose tissue [160]. Adiponectin action on the skeletal muscle is mediated by AMPK, which, in turn, is activated by adiponectin receptor 1 [161]. More recently, it has been demonstrated that the insulin sensitization induced by PPAR-γ ligands, depends on adiponectin [162]. Therefore, the role of adiponectin seems to be more vast and complex than first thought, including the regulation of energy expenditure and appetite [163]. Adiponectin appears to be in the pathway regulating insulin sensitivity and seems to distinguish between the "healthy obese" children and those at higher metabolic risk [164], and as such may in the future prove an effective marker of long-term risk [165]. Lower levels of adiponectin have been shown to predict future risk of MS over a 6-year period [166]. Low levels of adiponectin and increasing insulin resistance are also associated with the clinical features of MS [167]. Notably, low levels of adiponectin are associated with higher levels of inflammatory cytokines, whereas infusions of adiponectin in animal models result in a decrease in systemic inflammation from unclear mechanisms [158].

9.4. Chemerin

Chemerin is a novel adipokine that is produced by both adipose tissue and liver; moreover, it is a chemoattractant for immune cells such as macrophages and promotes adipocyte differentiation [168]. Chemerin levels have also been shown to be higher in obesity, some features of MS, diabetes, and NAFLD [168–170], and it appears to induce insulin resistance in skeletal muscle, the major site of peripheral insulin resistance [170]. Bremer *et al.* made the novel observation that both plasma and SAT levels of chemerin are higher in subjects with nascent MS, suggesting that chemerin could be involved early in the pathogenesis of the syndrome [171–173]. Another study in Caucasian subjects with MS (including some with concomitant diabetes), serum chemerin levels were reported to be significantly increased; however, they were not adjusted for adiposity [173]. In this study, the investigators did not find a correlation between insulin resistance, obtained by two measures (HOMA and the quantitative insulin sensitivity check index) and chemerin concentrations [174]. In a subsequent study in Korean subjects [175], the authors suggested that the ratio of chemerin to adiponectin might be a good predictor of MS, but did not report on adiposity-adjusted differences between patients with MS and controls. Also, Dong *et al.* reported

increased chemerin levels in patients with MS (41% on statin therapy) with and without CAD and suggested it was an independent predictor of angiographic CAD [176], but again they did not correct for adiposity compared to controls. Since VAT is not a major source of chemerin [177], studies from Bremer and other investigators [172,173] highlighted the contribution of SAT to circulating chemerin levels and its use as a potential biomarker of SAT dysregulation. Moreover, those findings demonstrate higher SAT and plasma chemerin concentrations independent of obesity in patients with MS but without T2D and CAD, and also confirm significant correlations with insulin resistance, inflammation, BP, and dyslipidemia, suggesting a potential role of chemerin in MS and its sequelae.

9.5. Omentin

Omentin mRNA is predominantly expressed in the stromal vascular fraction of VAT but is scarcely detectable in subcutaneous fat depots and mature adipocytes [178]. Omentin is found at lower levels in patients with glucose intolerance and diabetes [179]. While recombinant omentin enhances insulin-stimulated glucose uptake in adipose tissue, the molecular mechanism by which it achieves this beneficial effect remains to be worked out (reviewed in Ref. [180]).

9.6. Resistin

Resistin was discovered as an adipocytokine in animal models produced mainly by adipocytes, linking obesity with diabetes [30]. In humans, adipocytes seem to contribute only a small fraction of the resistin production [181] and inflammatory cells such as macrophages are considered the predominant source of circulating resistin [182]. Some studies have reported that resistin levels are increased in obese individuals [183,184], while others did not observe the phenomenon [185,186]. Population-based studies have shown that resistin levels are associated with metabolic impairments and insulin resistance [185,187,188], but the association between resistin levels and insulin sensitivity has been inconsistent in humans [183]. In children, resistin levels correlate with pubertal development but not with metabolic parameters [62]. Resistin levels have also been associated with coronary heart diseases [189] and were correlated with calcification deposition in coronary arteries [190]. In contrast, other studies have not shown a significant association between resistin and CADs [191,192]. Thus, the evidence linking

resistin with decreased insulin sensitivity or increased cardiovascular risk remains inconsistent.

Of note, the secretions of TNF-α, IL-6, and other cell adhesion molecules are increased by resistin [193]. An *in vitro* study demonstrated that resistin treatment increased the proliferation and migration of vascular smooth muscle and endothelial cells [194]. In summary, resistin may participate in cardiovascular physiopathology in humans via the action of macrophages implicated in the inflammatory response related to obesity.

9.7. Visfatin

Visfatin is also known as pre-B cell colony-enhancing factor or Nampt, since it is the limiting enzyme in nicotinamide adenine dinucleotide (NAD) biosynthesis [195]. Visfatin was first isolated from the visceral fat of humans and mice, but is also expressed in leukocytes, adipocytes, muscle cells, and hepatocytes [196–199]. In adipose tissue, however, it may be primarily the product of infiltrating macrophages as opposed to adipocytes. In CD14+ monocytes, visfatin induced the expression of IL-1β, TNF-α, IL-6, and other CD molecules [200].

While visfatin may frequently be upregulated in the obese state, its impact on insulin sensitivity and the underlying mechanisms are not clear yet. No correlation of plasma visfatin, or visfatin expression in subcutaneous, or VAT has been observed [201,202], although the serum visfatin levels were significantly reduced after gastric bypass surgery in morbidly obese subjects [203], also the serum visfatin levels are higher in patients with diabetes and diabetic nephropathy [204]. Visfatin is now regarded as an extracellular nicotinamide phosphoribosyltransferase (eNampt) enzyme and it seems to play an important role in insulin secretion from pancreatic β cell by systemic NAD biosynthesis [199]. *In vitro* and *in vivo*, visfatin mimics insulin action, but in human studies, it is paradoxically increased in disease conditions and shows correlation with systemic inflammation, vascular complications, and insulin secretion [200,205].

9.8. Retinol binding protein-4

Another factor released from adipocytes is retinol-binding protein-4, which appears to suppress the peripheral expression of glucose transporter-4 (GLUT-4)—a key glucose transporter in skeletal muscle—and is associated with an increased expression of monocyte chemoattractant protein-1 adipocytes [206–209]. Retinol-binding protein-4 (RBP4) represents another

untested potential marker for long-term risk [210]. Although the data are not always clear, some reports have noted increased levels of RBP4 in obese adolescents, corresponding to the amount of fat mass [211] and changes in insulin resistance [212]. The role of RBP4 in obesity and insulin resistance was first observed in 2005, when it was observed that RBP4 expression is increased in adipose tissue of mice selectively knocked down for GLUT-4 in the adipose tissue (adipose GLUT-$4^{-/-}$) and reduced in mice overexpressing GLUT-4 [207]. Moreover, not only were the RBP4 serum levels increased in other mouse models of obesity and insulin resistance [206] but also the transgenic overexpression of human RBP4 or its injection in normal mice caused insulin resistance. Therefore, it was suggested that RBP4 is an adipocyte-derived factor that affects insulin sensitivity in the muscle and liver [207]. After this observation, several clinical studies have tried to assess the role of the RBP4 in the pathogenesis of obesity-related insulin resistance. It has been shown, in fact, that RBP4 levels correlate with BMI [208], whole-body fat content and VAT content [213–216]. Moreover, RBP4 is preferentially expressed in visceral fat compared with subcutaneous fat [217]. Longitudinal observational and interventional studies have shown that a significant weight loss, achieved by diet, exercise, or bariatric surgery, leads to a decrease in circulating RBP4 levels as well as to a decreased expression of RBP4 in the adipose tissue [208]. The RBP4 levels are influenced not only by adiposity itself but also by the status of insulin resistance. In fact, RBP4 levels decrease after a weight-loss program in subjects with insulin resistance and T2D [208,218]; however, the decrease is specifically linked to those with improved insulin sensitivity [208] and, moreover, therapy with rosiglitazone (which is no longer available in commerce) also decreases circulating RBP4 levels [215]. Along with insulin resistance, circulating RBP4 levels were also found to be associated with other components and with the long-term complications of the MS [219,220], such as increased carotid intima-media thickness, cerebral infarction, early-onset cardiovascular disease, and increased risk of incident coronary disease [221]. While the role of RBP4 in obesity-related insulin resistance in youth seems to be well established [222], it does not seem to play any role in peripubertal insulin resistance [223]. Nevertheless, not all the observations concerning the association between RBP4 and adiposity seem to be consistent. Some studies, in fact, did not observe any association between RBP4 and BMI or T2D [224,225]. Those inconsistencies could be explained by the collection and dosage methods. In fact, plasma anticoagulants present in the tubes could alter the absolute amount of RBP4

immunoreactivity; moreover, the effect differs from subject to subject and, thus, glass vacuum tubes without additives are suggested as an optimal collection container. Differences in the immunoassays used can also influence the results, which is why it has been suggested to use quantitative Western blotting to full-length RBP4 protein to obtain an exact estimate of RBP4 levels [208].

9.9. Plasminogen activator inhibitor-1

In the study of Kraja *et al.*, plasminogen activator inhibitor-1 (PAI-1) represented an important contributor to the obesity and lipids factors and associated with the MS [226]. Similar to these findings, PAI-1 had a significant contribution in the "Metabolic syndrome" factor in Hanley *et al.* [227] and in the Tang *et al.* [228], and in the "Body mass" and lesser in the "insulin/glucose" factors in Sakkinen *et al.* studies [229]. PAI-1 was strongly associated with MS components such as BMI, TG, a homeostasis model assessment of insulin resistance, hs-CRP, and alanine aminotransferase [230]. PAI-1 prevents plasmin generation and is considered a primary inhibitor of fibrinolysis and of extracellular matrix degradation [231]. PAI-1 is lowered by weight loss and drugs that improve insulin sensitivity. PAI-deficient mice were resistant to diet-induced obesity, explained by increased energy expenditure [232]. PAI-1 is induced by many proinflammatory and pro-oxidant factors, for example, TNF-α [233,234]. Transgenic mice with elevated levels of PAI-1 in plasma developed venous occlusions [235], and when overexpressing human PAI-1, the mice develop macrovascular coronary thrombosis and subendocardial myocardial infarction [236]. Smith *et al.* [237] concluded that PAI-1 together with fibrinogen, DDIMER were associated significantly with risk of CVD. Consequently, PAI-1 has important functions and is a biomarker that can increase the prediction for MS in addition to the conventional risk factors. Mertens *et al.* [238] investigated the association of five biomarkers with the MS as defined by the NCEP criteria in 520 overweight and obese subjects and concluded that PAI-1 is a true component of MS. However, questions remain open whether PAI-1 genes contribute to the MS development or represent a step in the cascade of the biochemical pathways of MS. It has been proposed that as result of obesity and hypoxia WAT overspills inflammation markers [239]. Skurk and Hauner [240] reviewed obesity and impaired fibrinolysis and reported that increased fat cell size and adipose tissue mass associate with higher offerings of PAI-1 in circulation. Moreover, Liang *et al.* [232] studying cultured adipocytes from

PAI-1(+/+) and PAI-1(−/−) mice found that PAI-1 deficiency had a protective role against insulin resistance, and was also associated with up- and downregulation of some other important genes.

9.10. Pigment epithelium-derived factor

Pigment epithelium-derived factor (PEDF) is a glycoprotein of approximately 50 kDa, mainly expressed in the adipose tissue and liver, but initially observed in retinal epithelial cells [241,242]. PEDF is an important determinant of oxidative stress [243,244], inflammation, and angiogenesis [245,246]. The major source of PEDF seems to be adipose tissue; in fact, there is a strong relationship between PEDF and measures of adiposity [247].

Although the association between PEDF, insulin resistance, and the MS has been shown in humans [247–249], most of the mechanistic studies dealing with the relationship between PEDF and insulin resistance have been performed in animal models [241]. In particular, the administration of PEDF in lean mice dramatically increases insulin resistance [241], probably through an increase of plasma FFA, skeletal muscle, and liver triacylglycerol content, as well as through the accumulation of the fatty acid metabolites, ceramide, and DAG, in skeletal muscle [241]. Moreover, when a PEDF-neutralizing antibody is administered to obese mice, insulin sensitivity increases, whereas skeletal muscle and liver triacylglycerol content and skeletal muscle ceramide and DAG decrease [241]. These observations suggest that one of the mechanisms by which increased PEDF levels may lead to developing insulin resistance is linked to the excessive basal adipose tissue lipolysis and consequent increase of FFAs in the blood, which, in turn, causes ectopic lipid deposition in skeletal muscle and liver.

Recent studies, in fact, have identified a triglyceride lipase (ATGL/PNPLA2) as a putative receptor for PEDF [250] and as a ligand for endothelial cell-surface $F_{(1)}F_{(0)}$-ATP synthase [251]; however, there is no experimental evidence that PEDF can exert its effect on insulin resistance through these receptors. Therefore, while it is now clear that PEDF is a biomarker for the MS, future studies need to address the mechanism of action by which PEDF modulates insulin resistance and the potential clinical implications.

10. ADIPOCYTE LIPID-DROPLET MOLECULES

10.1. Perilipin

Obesity may play a major role in triggering the MS by interacting with genetic variants in candidate genes for dyslipidemia, hypertension, and

insulin resistance. In support of this hypothesis, several studies at several candidate genes, mainly adipokines and perilipin (*PLIN*), have already demonstrated the significance of these interactions [252]. Common polymorphisms in the *PLIN* gene modulate the response of insulin sensibility to changes in dietary saturated fat and carbohydrates [117]. Humans, heterozygote for a truncated form of Plin1, display partial lipodystrophy, severe dyslipidemia, and insulin-resistant diabetes [253].

Lipid droplets are coated with one or more of five members of the perilipin family of proteins: adipophilin, TIP47, OXPAT/MLDP, S3-12, and perilipin. The most studied member of the family, perilipin (Plin1), is the most abundant protein on the surfaces of adipocyte lipid droplets and the major substrate for cAMP-dependent protein kinase (protein kinase A—PKA) in lipolytically stimulated adipocytes [254]. Perilipin serves important functions in the regulation of basal and hormonally stimulated lipolysis. Under basal conditions, perilipin restricts the access of cytosolic lipases to lipid droplets and thus promotes triacylglycerol storage. In times of energy deficit, perilipin is phosphorylated by PKA and facilitates maximal lipolysis by hormone-sensitive lipase (HSL) and adipose triglyceride lipase [255]. Perilipin probably serves as a dynamic scaffold to coordinate the access of enzymes to the lipid droplet in a manner that is responsive to the metabolic status of the adipocyte. Plin1 interacts with other proteins to balance lipid storage and hydrolysis such as caveolin-1, which is a component of caveolae and is highly enriched in adipose cells where it could regulate fatty acids trafficking and accumulation [256,257].

10.2. Cell death-inducing DFF45-like effector

The cell death-inducing DFF45-like effector (CIDE) proteins, CIDE-A, CIDE-B, and CIDE-C have distinct tissue expression patterns with CIDE-A in brown adipose tissue (BAT) in rodents [258], CIDE-B [259] in liver, and CIDE-C in WAT and BAT [260,261]. Interestingly, mRNAs of Fsp27 (rodent homolog of CIDE-C) and CIDE-A were also detected in fatty livers in which excess amount of lipid is accumulated and large lipid droplets are formed [262–264]. CIDE proteins are subjected to tight regulation on both transcriptional and posttranslational levels, for example, CICE-C is induced by PPAR-γ2 [265]. Studies using animals deficient in CIDE proteins have demonstrated that this class of proteins plays important roles in lipid storage, lipid droplet formation, lipolysis, and the development of obesity, diabetes, and liver steatosis. $Fsp27^{-/-}$ mice also have dramatically lower levels of TAG and much smaller lipid droplets in their

white adipocytes and are protected from diet-induced obesity. *Fsp27*$^{-/-}$ mice also have higher glucose uptake rates and improved insulin sensitivity [260,261]. Furthermore, *Fsp27* deficiency leads to a reduction in fat accumulation and improved insulin sensitivity in *leptin*-deficient *ob/ob* mice. The increased insulin sensitivity in *Fsp27*$^{-/-}$ mice is likely due to increased expression and phosphorylation of crucial factors such as GLUT-4, IRS-1, and AKT2 in insulin signaling pathway in the WAT. The *Fsp27*$^{-/-}$ mice also have higher lipolysis rates, especially the basal level. Interestingly, *Fsp27*$^{-/-}$ WAT tends to acquire properties of BAT, such as smaller lipid droplets, increased mitochondrial volume and activity, and enhanced expression of BAT-specific genes such as Ucp1, CIDE-A, PPAR-α, and Dio2. The attainment of BAT-like property in the WAT of *Fsp27*$^{-/-}$ mice is likely due to increased levels of key metabolic regulators such as FoxC2, PPAR, and PGC-1α, as well as reduced expression levels of Rb, p107, and RIP140 [266].

In WAT of BMI-matched obese humans, levels of CIDE-A and CIDE-C are positively correlated with insulin sensitivity indicating their role in controling adipose lipolysis and thus circulating fatty acids [267]. Furthermore, levels of CIDE-C were reduced in response to caloric restriction in obese patients [268]. Overall, these data suggest that CIDE proteins are novel regulators of the development of metabolic diseases, such as obesity, T2D, and liver steatosis.

10.3. AMP-activated protein kinase and Sirtuin 1

AMPK has been implicated in regulating a variety of cellular functions including energy state, fuel metabolism, mitochondrial biogenesis, protein and ceramide synthesis, cell growth, and proliferation. In addition, its activation was initially shown to inhibit glucose, palmitate, and TNF-α-induced inflammation, insulin resistance, apoptosis, and oxidative stress *in vitro* [269,270] and later in other cells [271]. Decreased AMPK activity has been observed in obese and insulin-resistant rodent models with MS- associated disorders including *ob/ob* and *db/db* mice, Zucker Diabetic Fatty (ZDF) rats, and fat-fed rodents [271]. Pharmacological AMPK activation in the leptin-resistant ZDF rat has been shown to diminish ectopic lipid deposition, inflammation, and apoptosis in pancreatic islets, therefore preventing the development of diabetes [272]. In humans, AMPK activity is diminished in omental, subcutaneous, and epiploic fat of severely obese insulin-resistant compared to equally obese insulin-sensitive individuals. AMPK activation has been shown to inhibit inflammasome formation in macrophages

[273,274] as well as the conversion of monocytes to M1 macrophages [275]. Oxidative stress [276] and inflammation as well as fatty acids have been shown to diminish AMPK activity by their effects on protein phosphatases [277] in cultured cells and rodents *in vivo*. In humans, decreases in AMPK activity and increases in oxidative stress appear to be present in multiple fat depots of insulin-resistant patients, whereas changes in the expression of genes for specific inflammatory cells and other molecules (e.g., PGC-1α, various cytokines and chemokines, and adhesion molecules) are more depot specific [106]. Taken together, these data suggest that the decrease in AMPK activity plays a key pathogenetic role in the development of the MS.

Sirtuins, a family of histone protein deacetylases that have long been linked to the antiaging effect of caloric restriction in rodents and other species [278] can activate AMPK and *vice versa* and have many actions and target molecules (e.g., PGC-1α, FOXO, and p53) in common with the AMPK [271]. As for AMPK, downregulation of SIRT1 in adipose tissue has been shown to increase obesity, macrophage accumulation, and inflammation in rodent fat [108,279] while activation of both molecules has been shown to diminish inflammation in macrophages [275,280]. Gillum *et al*. showed that the SIRT1 expression in adipose tissue of obese insulin-resistant humans is associated with increased macrophage infiltration [108]. As already noted, AMPK has been reported to inhibit inflammasome formation by effects on mitophagy and oxidative stress [274]. In contrast, SIRT1 is cleaved and inactivated by caspase-1, a member of the NLRP3 inflammasome [279]. Furthermore, decreases in the activity of both AMPK and SIRT1 have been linked to the development of obesity and insulin resistance in a wide variety of rodent models [271,279,281]. In fat-fed obese rats, increases in AMPK activity and SIRT1 abundance have been observed after gastric bypass surgery [282].

11. INFLAMMATION MARKERS

Since the MS is thought to be associated with a chronic low-grade inflammation, a growing body of evidence suggests more and more inflammatory markers to be closely related to the MS and its consequences. For example, circulating levels of IL-18 have been reported to be elevated in subjects with the MS, to be closely associated with the components of the syndrome, to predict cardiovascular events and mortality in populations with the MS, and to precede the development of T2D [283]. The most

important inflammation marker in circulation, CRP, IL-6, and TNF-α will be discussed in more detail below.

11.1. C-reactive protein

CRP is the most commonly used marker to assess systemic inflammation. CRP is produced by the liver, peripheral leukocytes, and even the adipose tissue in response to multiple cues, particularly increases in IL-6, and other systemic inflammatory cytokines [284,285]. In the periphery, CRP has specific roles including the activation of phagocytic cells—at least in part by binding to the Fc-γ-RIIa receptor [286]. While longitudinal data regarding the predictive importance of CRP in adolescents are lacking, cross-sectional data support the potential for such a relationship. As in adults, CRP levels in childhood are increased in the settings of obesity and are strongly associated with waist circumference, BMI, and adiposity [287–291]. With respect to insulin resistance, levels of CRP levels are strongly associated with measurements of insulin resistance (such as fasting insulin and the homeostasis model of insulin resistance) and are higher in overweight adolescents with insulin resistance versus without insulin resistance as assessed by insulin clamp [288–291]. CRP levels are higher in adolescents with MS than in those without MS [288,289,292]. Perhaps most importantly, in a cohort of 2195 individuals who had CRP measured at 3–18 years of age, CRP levels predicted the presence of MS as adults 21 years later [293]. In addition, CRP is independently associated with carotid artery media thickness in adolescence, an early finding related to atherosclerotic plaque formation [294]—although this has not been demonstrated in all studies on the topic [295].

11.2. Tumor necrosis factor-alpha and interleukin-6

TNF-α and IL-6 are upregulated in adipocytes undergoing proinflammatory stimulation in the obese state. While TNF-α acts predominantly locally and cannot be measured at elevated levels in circulation under metabolically challenged conditions, IL-6 is released effectively from adipose tissue. In the case of visceral adipocytes, IL-6 is released into the portal vein where it is shunted directly into the liver [296] and induces CRP production in hepatocytes [297]. In adolescents, IL-6 plasma levels are inversely related to physical activity, independently of adiposity, and fat localization [298]. A number of studies showed that obese children, compared to lean controls, were more insulin resistant and had significantly higher TNF-α levels, which

were negatively correlated to HDL and positively correlated to TG [299,300]. Furthermore, children of parents having MS also showed higher levels of TNF-α, whereas their HDL cholesterol concentrations were lower compared with those of age- and sex-matched control subjects [301].

ACKNOWLEDGMENTS

This work was supported by the American Heart Association (AHA) (13SDG14640038) and 2012 Yale Center for Clinical Investigation (YCCI) scholar award to N. S. This publication was also made possible by CTSA Grant Number UL1 RR024139 from the National Center for Advancing Translational Science (NCATS), a component of the National Institutes of Health (NIH), and NIH roadmap for Medical Research. Its contents are solely the responsibility of the authors and do not necessarily represent the official view of NIH.

REFERENCES

[1] M. de Onis, M. Blossner, E. Borghi, Global prevalence and trends of overweight and obesity among preschool children, Am. J. Clin. Nutr. 92 (2010) 1257–1264.
[2] G.M. Reaven, Banting lecture 1988. Role of insulin resistance in human disease, Diabetes 37 (1988) 1595–1607.
[3] R. Weiss, S.E. Taksali, S. Dufour, C.W. Yeckel, X. Papademetris, G. Cline, W.V. Tamborlane, J. Dziura, G.I. Shulman, S. Caprio, The "obese insulin-sensitive" adolescent: importance of adiponectin and lipid partitioning, J. Clin. Endocrinol. Metab. 90 (2005) 3731–3737.
[4] R. Weiss, J. Dziura, T.S. Burgert, W.V. Tamborlane, S.E. Taksali, C.W. Yeckel, K. Allen, M. Lopes, M. Savoye, J. Morrison, R.S. Sherwin, S. Caprio, Obesity and the metabolic syndrome in children and adolescents, N. Engl. J. Med. 350 (2004) 2362–2374.
[5] T. Galbo, G.I. Shulman, Lipid-induced hepatic insulin resistance, Aging 5 (2013) 582–583.
[6] V.T. Samuel, K.F. Petersen, G.I. Shulman, Lipid-induced insulin resistance: unravelling the mechanism, Lancet 375 (2010) 2267–2277.
[7] P. Zimmet, G. Alberti, F. Kaufman, N. Tajima, M. Silink, S. Arslanian, G. Wong, P. Bennett, J. Shaw, S. Caprio, International Diabetes Federation Task Force on Epidemiology and Prevention of,Diabetes, The metabolic syndrome in children and adolescents, Lancet 369 (2007) 2059–2061.
[8] E. Goodman, C. Li, Y.K. Tu, E. Ford, S.S. Sun, T.T. Huang, Stability of the factor structure of the metabolic syndrome across pubertal development: confirmatory factor analyses of three alternative models, J. Pediatr. 155 (2009) S5.e1–S5.e8.
[9] Obesity: preventing and managing the global epidemic. Report of a WHO consultation, World Health Organ. Tech. Rep. Ser. 894 (2000) i–xii, 1–253.
[10] Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults—The Evidence Report, 6 National Institutes of Health, Obesity Research, 1998, pp. 51S–209S, Suppl. 2.
[11] T.J. Cole, M.C. Bellizzi, K.M. Flegal, W.H. Dietz, Establishing a standard definition for child overweight and obesity worldwide: international survey, BMJ 320 (2000) 1240–1243.
[12] J.H. Himes, W.H. Dietz, Guidelines for overweight in adolescent preventive services: recommendations from an expert committee. The Expert Committee on Clinical

Guidelines for Overweight in Adolescent Preventive Services, Am. J. Clin. Nutr. 59 (1994) 307–316.
[13] R.J. Kuczmarski, C.L. Ogden, S.S. Guo, L.M. Grummer-Strawn, K.M. Flegal, Z. Mei, R. Wei, L.R. Curtin, A.F. Roche, C.L. Johnson, 2000 CDC Growth Charts for the United States: methods and development, Vital Health Stat. 11 (2002) 1–190, Data from the national health survey.
[14] J.R. Fernandez, D.T. Redden, A. Pietrobelli, D.B. Allison, Waist circumference percentiles in nationally representative samples of African-American, European-American, and Mexican-American children and adolescents, J. Pediatr. 145 (2004) 439–444.
[15] I. Janssen, P.T. Katzmarzyk, S.R. Srinivasan, W. Chen, R.M. Malina, C. Bouchard, G.S. Berenson, Combined influence of body mass index and waist circumference on coronary artery disease risk factors among children and adolescents, Pediatrics 115 (2005) 1623–1630.
[16] M.C. Pouliot, J.P. Despres, S. Lemieux, S. Moorjani, C. Bouchard, A. Tremblay, A. Nadeau, P.J. Lupien, Waist circumference and abdominal sagittal diameter: best simple anthropometric indexes of abdominal visceral adipose tissue accumulation and related cardiovascular risk in men and women, Am. J. Cardiol. 73 (1994) 460–468.
[17] P.T. Katzmarzyk, Waist circumference percentiles for Canadian youth 11–18 y of age, Eur. J. Clin. Nutr. 58 (2004) 1011–1015.
[18] R. Zannolli, G. Morgese, Waist percentiles: a simple test for atherogenic disease? Acta Paediatr. 85 (1996) 1368–1369.
[19] H.D. McCarthy, K.V. Jarrett, H.F. Crawley, The development of waist circumference percentiles in British children aged 5.0–16.9 y, Eur. J. Clin. Nutr. 55 (2001) 902–907.
[20] C.J. Dobbelsteyn, M.R. Joffres, D.R. MacLean, G. Flowerdew, A comparative evaluation of waist circumference, waist-to-hip ratio and body mass index as indicators of cardiovascular risk factors. The Canadian Heart Health Surveys, Int. J. Obes. Relat. Metab. Disord. 25 (2001) 652–661.
[21] I. Janssen, P.T. Katzmarzyk, R. Ross, Body mass index, waist circumference, and health risk: evidence in support of current National Institutes of Health guidelines, Arch. Intern. Med. 162 (2002) 2074–2079.
[22] I. Janssen, S.B. Heymsfield, D.B. Allison, D.P. Kotler, R. Ross, Body mass index and waist circumference independently contribute to the prediction of nonabdominal, abdominal subcutaneous, and visceral fat, Am. J. Clin. Nutr. 75 (2002) 683–688.
[23] R.E. Van Pelt, E.M. Evans, K.B. Schechtman, A.A. Ehsani, W.M. Kohrt, Waist circumference vs body mass index for prediction of disease risk in postmenopausal women, Int. J. Obes. Relat. Metab. Disord. 25 (2001) 1183–1188.
[24] C. Maffeis, A. Pietrobelli, A. Grezzani, S. Provera, L. Tato, Waist circumference and cardiovascular risk factors in prepubertal children, Obes. Res. 9 (2001) 179–187.
[25] D.S. Freedman, M.K. Serdula, S.R. Srinivasan, G.S. Berenson, Relation of circumferences and skinfold thicknesses to lipid and insulin concentrations in children and adolescents: the Bogalusa Heart Study, Am. J. Clin. Nutr. 69 (1999) 308–317.
[26] S.C. Savva, M. Tornaritis, M.E. Savva, Y. Kourides, A. Panagi, N. Silikiotou, C. Georgiou, A. Kafatos, Waist circumference and waist-to-height ratio are better predictors of cardiovascular disease risk factors in children than body mass index, Int. J. Obes. Relat. Metab. Disord. 24 (2000) 1453–1458.
[27] W.Y. Fujimoto, R.W. Bergstrom, E.J. Boyko, K.W. Chen, D.L. Leonetti, L. Newell-Morris, J.B. Shofer, P.W. Wahl, Visceral adiposity and incident coronary heart disease in Japanese-American men. The 10-year follow-up results of the Seattle Japanese-American Community Diabetes Study, Diabetes Care 22 (1999) 1808–1812.
[28] I.M. O'Shaughnessy, T.J. Myers, K. Stepniakowski, P. Nazzaro, T.M. Kelly, R.G. Hoffmann, B.M. Egan, A.H. Kissebah, Glucose metabolism in abdominally obese hypertensive and normotensive subjects, Hypertension 26 (1995) 186–192.

[29] J.R. Williamson, R.A. Kreisberg, P.W. Felts, Mechanism for the stimulation of gluconeogenesis by fatty acids in perfused rat liver, Proc. Natl. Acad. Sci. U.S.A. 56 (1966) 247–254.
[30] C.M. Steppan, S.T. Bailey, S. Bhat, E.J. Brown, R.R. Banerjee, C.M. Wright, H.R. Patel, R.S. Ahima, M.A. Lazar, The hormone resistin links obesity to diabetes, Nature 409 (2001) 307–312.
[31] G.S. Hotamisligil, P. Peraldi, A. Budavari, R. Ellis, M.F. White, B.M. Spiegelman, IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance, Science 271 (1996) 665–668.
[32] M. Ashwell, P. Gunn, S. Gibson, Waist-to-height ratio is a better screening tool than waist circumference and BMI for adult cardiometabolic risk factors: systematic review and meta-analysis, Obes. Rev. 13 (2012) 275–286.
[33] L. Graves, S.P. Garnett, C.T. Cowell, L.A. Baur, A. Ness, N. Sattar, D.A. Lawlor, Waist-to-height ratio and cardiometabolic risk factors in adolescence: findings from a prospective birth cohort, Pediatr. Obes. (2013) doi: 10.1111/j.2047-6310.2013.00192.x.
[34] M. Teran-Garcia, C. Bouchard, Genetics of the metabolic syndrome, Appl. Physiol. Nutr. Metab. 32 (2007) 89–114.
[35] A.T. Kraja, S.C. Hunt, J.S. Pankow, R.H. Myers, G. Heiss, C.E. Lewis, D. Rao, M.A. Province, An evaluation of the metabolic syndrome in the HyperGEN study, Nutr. Metab. 2 (2005) 2.
[36] R. Weiss, M. Shaw, M. Savoye, S. Caprio, Obesity dynamics and cardiovascular risk factor stability in obese adolescents, Pediatr. Diabetes 10 (2009) 360–367.
[37] L. Rafalson, T.H. Pham, S.M. Willi, M. Marcus, A. Jessup, T. Baranowski, The association between acanthosis nigricans and dysglycemia in an ethnically diverse group of eighth grade students, Obesity (Silver Spring) 21 (2013) E328–E333.
[38] N. Santoro, A. Amato, A. Grandone, C. Brienza, P. Savarese, N. Tartaglione, P. Marzuillo, L. Perrone, E. Miraglia Del Giudice, Predicting metabolic syndrome in obese children and adolescents: look, measure and ask, Obes. Facts 6 (2013) 48–56.
[39] L. Taittonen, M. Uhari, M. Nuutinen, J. Turtinen, T. Pokka, H.K. Akerblom, Insulin and blood pressure among healthy children. Cardiovascular risk in young Finns, Am. J. Hypertens. 9 (1996) 194–199.
[40] W. Bao, S.R. Srinivasan, G.S. Berenson, Persistent elevation of plasma insulin levels is associated with increased cardiovascular risk in children and young adults. The Bogalusa Heart Study, Circulation 93 (1996) 54–59.
[41] L. Landsberg, Hyperinsulinemia: possible role in obesity-induced hypertension, Hypertension 19 (1992) I61–I66.
[42] R.A. DeFronzo, C.R. Cooke, R. Andres, G.R. Faloona, P.J. Davis, The effect of insulin on renal handling of sodium, potassium, calcium, and phosphate in man, J. Clin. Invest. 55 (1975) 845–855.
[43] R.W. Stout, E.L. Bierman, R. Ross, Effect of insulin on the proliferation of cultured primate arterial smooth muscle cells, Circ. Res. 36 (1975) 319–327.
[44] F. Assimacopoulos-Jeannet, S. Brichard, F. Rencurel, I. Cusin, B. Jeanrenaud, In vivo effects of hyperinsulinemia on lipogenic enzymes and glucose transporter expression in rat liver and adipose tissues, Metab. Clin. Exp. 44 (1995) 228–233.
[45] K.L. Donnelly, C.I. Smith, S.J. Schwarzenberg, J. Jessurun, M.D. Boldt, E.J. Parks, Sources of fatty acids stored in liver and secreted via lipoproteins in patients with non-alcoholic fatty liver disease, J. Clin. Invest. 115 (2005) 1343–1351.
[46] K.F. Petersen, S. Dufour, D.B. Savage, S. Bilz, G. Solomon, S. Yonemitsu, G.W. Cline, D. Befroy, L. Zemany, B.B. Kahn, X. Papademetris, D.L. Rothman, G.I. Shulman, The role of skeletal muscle insulin resistance in the pathogenesis of the metabolic syndrome, Proc. Natl. Acad. Sci. U.S.A. 104 (2007) 12587–12594.

[47] S.A. Amiel, R.S. Sherwin, D.C. Simonson, A.A. Lauritano, W.V. Tamborlane, Impaired insulin action in puberty. A contributing factor to poor glycemic control in adolescents with diabetes, N. Engl. J. Med. 315 (1986) 215–219.
[48] S.A. Amiel, W.V. Tamborlane, D.C. Simonson, R.S. Sherwin, Defective glucose counterregulation after strict glycemic control of insulin-dependent diabetes mellitus, N. Engl. J. Med. 316 (1987) 1376–1383.
[49] G.S. Hotamisligil, Inflammation and metabolic disorders, Nature 444 (2006) 860–867.
[50] American Diabetes Association, Diagnosis and classification of diabetes mellitus, Diabetes Care 33 (Suppl. 1) (2010) S62–S69.
[51] A.M. Cali, R.C. Bonadonna, M. Trombetta, R. Weiss, S. Caprio, Metabolic abnormalities underlying the different prediabetic phenotypes in obese adolescents, J. Clin. Endocrinol. Metab. 93 (2008) 1767–1773.
[52] C.L. Rohlfing, R.R. Little, H.M. Wiedmeyer, J.D. England, R. Madsen, M.I. Harris, K.M. Flegal, M.S. Eberhardt, D.E. Goldstein, Use of GHb (HbA1c) in screening for undiagnosed diabetes in the U.S. population, Diabetes Care 23 (2000) 187–191.
[53] A.D. Pradhan, N. Rifai, J.E. Buring, P.M. Ridker, Hemoglobin A1c predicts diabetes but not cardiovascular disease in nondiabetic women, Am. J. Med. 120 (2007) 720–727.
[54] E. Selvin, M.W. Steffes, H. Zhu, K. Matsushita, L. Wagenknecht, J. Pankow, J. Coresh, F.L. Brancati, Glycated hemoglobin, diabetes, and cardiovascular risk in nondiabetic adults, N. Engl. J. Med. 362 (2010) 800–811.
[55] P. Nowicka, N. Santoro, H. Liu, D. Lartaud, M.M. Shaw, R. Goldberg, C. Guandalini, M. Savoye, P. Rose, S. Caprio, Utility of hemoglobin A (1c) for diagnosing prediabetes and diabetes in obese children and adolescents, Diabetes Care 34 (2011) 1306–1311.
[56] R. Weiss, S. Dufour, S.E. Taksali, W.V. Tamborlane, K.F. Petersen, R.C. Bonadonna, L. Boselli, G. Barbetta, K. Allen, F. Rife, M. Savoye, J. Dziura, R. Sherwin, G.I. Shulman, S. Caprio, Prediabetes in obese youth: a syndrome of impaired glucose tolerance, severe insulin resistance, and altered myocellular and abdominal fat partitioning, Lancet 362 (2003) 951–957.
[57] C. Giannini, R. Weiss, A. Cali, R. Bonadonna, N. Santoro, B. Pierpont, M. Shaw, S. Caprio, Evidence for early defects in insulin sensitivity and secretion before the onset of glucose dysregulation in obese youths: a longitudinal study, Diabetes 61 (2012) 606–614.
[58] R. Weiss, S. Caprio, M. Trombetta, S.E. Taksali, W.V. Tamborlane, R. Bonadonna, Beta-cell function across the spectrum of glucose tolerance in obese youth, Diabetes 54 (2005) 1735–1743.
[59] A.M. Cali, C.D. Man, C. Cobelli, J. Dziura, A. Seyal, M. Shaw, K. Allen, S. Chen, S. Caprio, Primary defects in beta-cell function further exacerbated by worsening of insulin resistance mark the development of impaired glucose tolerance in obese adolescents, Diabetes Care 32 (2009) 456–461.
[60] S.L. Edelstein, W.C. Knowler, R.P. Bain, R. Andres, E.L. Barrett-Connor, G.K. Dowse, S.M. Haffner, D.J. Pettitt, J.D. Sorkin, D.C. Muller, V.R. Collins, R.F. Hamman, Predictors of progression from impaired glucose tolerance to NIDDM: an analysis of six prospective studies, Diabetes 46 (1997) 701–710.
[61] M.F. Saad, W.C. Knowler, D.J. Pettitt, R.G. Nelson, D.M. Mott, P.H. Bennett, The natural history of impaired glucose tolerance in the Pima Indians, N. Engl. J. Med. 319 (1988) 1500–1506.
[62] N. Gungor, S. Arslanian, Progressive beta cell failure in type 2 diabetes mellitus of youth, J. Pediatr. 144 (2004) 656–659.
[63] C. Giannini, C.D. Man, L. Groop, C. Cobelli, H. Zhao, M.M. Shaw, E. Duran, B. Pierpont, A.E. Bale, S. Caprio, N. Santoro, The co-occurrence of risk alleles in

or near genes modulating insulin secretion predisposes obese youth to prediabetes, Diabetes Care 37 (2) (2014) 475–482.
[64] J.B. Schwimmer, R. Deutsch, J.B. Rauch, C. Behling, R. Newbury, J.E. Lavine, Obesity, insulin resistance, and other clinicopathological correlates of pediatric non-alcoholic fatty liver disease, J. Pediatr. 143 (2003) 500–505.
[65] Z.M. Younossi, M. Stepanova, N. Rafiq, H. Makhlouf, Z. Younoszai, R. Agrawal, Z. Goodman, Pathologic criteria for nonalcoholic steatohepatitis: interprotocol agreement and ability to predict liver-related mortality, Hepatology 53 (2011) 1874–1882.
[66] A.E. Feldstein, P. Charatcharoenwitthaya, S. Treeprasertsuk, J.T. Benson, F.B. Enders, P. Angulo, The natural history of non-alcoholic fatty liver disease in children: a follow-up study for up to 20 years, Gut 58 (2009) 1538–1544.
[67] T.S. Burgert, S.E. Taksali, J. Dziura, T.R. Goodman, C.W. Yeckel, X. Papademetris, R.T. Constable, R. Weiss, W.V. Tamborlane, M. Savoye, A.A. Seyal, S. Caprio, Alanine aminotransferase levels and fatty liver in childhood obesity: associations with insulin resistance, adiponectin, and visceral fat, J. Clin. Endocrinol. Metab. 91 (2006) 4287–4294.
[68] A.M. Cali, A.M. De Oliveira, H. Kim, S. Chen, M. Reyes-Mugica, S. Escalera, J. Dziura, S.E. Taksali, R. Kursawe, M. Shaw, M. Savoye, B. Pierpont, R.T. Constable, S. Caprio, Glucose dysregulation and hepatic steatosis in obese adolescents: is there a link? Hepatology 49 (2009) 1896–1903.
[69] E. D'Adamo, V. Northrup, R. Weiss, N. Santoro, B. Pierpont, M. Savoye, G. O'Malley, S. Caprio, Ethnic differences in lipoprotein subclasses in obese adolescents: importance of liver and intraabdominal fat accretion, Am. J. Clin. Nutr. 92 (2010) 500–508.
[70] E. D'Adamo, A.M. Cali, R. Weiss, N. Santoro, B. Pierpont, V. Northrup, S. Caprio, Central role of fatty liver in the pathogenesis of insulin resistance in obese adolescents, Diabetes Care 33 (2010) 1817–1822.
[71] G. Kim, C. Giannini, B. Pierpont, A.E. Feldstein, N. Santoro, R. Kursawe, M. Shaw, E. Duran, R. Goldberg, J. Dziura, S. Caprio, Longitudinal effects of MRI-measured hepatic steatosis on biomarkers of glucose homeostasis and hepatic apoptosis in obese youth, Diabetes Care 36 (2013) 130–136.
[72] N. Santoro, A.E. Feldstein, E. Enoksson, B. Pierpont, R. Kursawe, G. Kim, S. Caprio, The association between hepatic fat content and liver injury in obese children and adolescents: effects of ethnicity, insulin resistance, and common gene variants, Diabetes Care 36 (2013) 1353–1360.
[73] R. Guerrero, G.L. Vega, S.M. Grundy, J.D. Browning, Ethnic differences in hepatic steatosis: an insulin resistance paradox? Hepatology 49 (2009) 791–801.
[74] A.E. Feldstein, S.M. Bailey, Emerging role of redox dysregulation in alcoholic and nonalcoholic fatty liver disease, Antioxid. Redox Signal. 15 (2011) 421–424.
[75] N. Chalasani, M.A. Deeg, D.W. Crabb, Systemic levels of lipid peroxidation and its metabolic and dietary correlates in patients with nonalcoholic steatohepatitis, Am. J. Gastroenterol. 99 (2004) 1497–1502.
[76] Z. Yesilova, H. Yaman, C. Oktenli, A. Ozcan, A. Uygun, E. Cakir, S.Y. Sanisoglu, A. Erdil, Y. Ates, M. Aslan, U. Musabak, M.K. Erbil, N. Karaeren, K. Dagalp, Systemic markers of lipid peroxidation and antioxidants in patients with nonalcoholic fatty liver disease, Am. J. Gastroenterol. 100 (2005) 850–855.
[77] H. Cortez-Pinto, L. Jesus, H. Barros, C. Lopes, M.C. Moura, M.E. Camilo, How different is the dietary pattern in non-alcoholic steatohepatitis patients? Clin. Nutr. 25 (2006) 816–823.
[78] K. Toshimitsu, B. Matsuura, I. Ohkubo, T. Niiya, S. Furukawa, Y. Hiasa, M. Kawamura, K. Ebihara, M. Onji, Dietary habits and nutrient intake in non-alcoholic steatohepatitis, Nutrition 23 (2007) 46–52.

[79] J. Araya, R. Rodrigo, L.A. Videla, L. Thielemann, M. Orellana, P. Pettinelli, J. Poniachik, Increase in long-chain polyunsaturated fatty acid n-6/n-3 ratio in relation to hepatic steatosis in patients with non-alcoholic fatty liver disease, Clin. Sci. (Lond.) 106 (2004) 635–643.
[80] Y. Li, D. Chen, The optimal dose of omega-3 supplementation for non-alcoholic fatty liver disease, J. Hepatol. 57 (2012) 468–469, author reply 469–470.
[81] P. Puri, R.A. Baillie, M.M. Wiest, F. Mirshahi, J. Choudhury, O. Cheung, C. Sargeant, M.J. Contos, A.J. Sanyal, A lipidomic analysis of nonalcoholic fatty liver disease, Hepatology 46 (2007) 1081–1090.
[82] N. Santoro, S. Caprio, C. Giannini, G. Kim, R. Kursawe, B. Pierpont, M.M. Shaw, A.E. Feldstein, Oxidized fatty acids: a potential pathogenic link between fatty liver and type 2 diabetes in obese adolescents? Antioxid Redox Signal 20 (2) (2014) 383–389.
[83] G. Krey, O. Braissant, F. L'Horset, E. Kalkhoven, M. Perroud, M.G. Parker, W. Wahli, Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of peroxisome proliferator-activated receptors by coactivator-dependent receptor ligand assay, Mol. Endocrinol. 11 (1997) 779–791.
[84] S. Romeo, J. Kozlitina, C. Xing, A. Pertsemlidis, D. Cox, L.A. Pennacchio, E. Boerwinkle, J.C. Cohen, H.H. Hobbs, Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease, Nat. Genet. 40 (2008) 1461–1465.
[85] L. Valenti, A. Alisi, E. Galmozzi, A. Bartuli, B. Del Menico, A. Alterio, P. Dongiovanni, S. Fargion, V. Nobili, I148M patatin-like phospholipase domain-containing 3 gene variant and severity of pediatric nonalcoholic fatty liver disease, Hepatology 52 (2010) 1274–1280.
[86] L. Valenti, A. Al-Serri, A.K. Daly, E. Galmozzi, R. Rametta, P. Dongiovanni, V. Nobili, E. Mozzi, G. Roviaro, E. Vanni, E. Bugianesi, M. Maggioni, A.L. Fracanzani, S. Fargion, C.P. Day, Homozygosity for the patatin-like phospholipase-3/adiponutrin I148M polymorphism influences liver fibrosis in patients with nonalcoholic fatty liver disease, Hepatology 51 (2010) 1209–1217.
[87] N. Santoro, R. Kursawe, E. D'Adamo, D.J. Dykas, C.K. Zhang, A.E. Bale, A.M. Cali, D. Narayan, M.M. Shaw, B. Pierpont, M. Savoye, D. Lartaud, S. Eldrich, S.W. Cushman, H. Zhao, G.I. Shulman, S. Caprio, A common variant in the patatin-like phospholipase 3 gene (PNPLA3) is associated with fatty liver disease in obese children and adolescents, Hepatology 52 (2010) 1281–1290.
[88] M. Graff, K.E. North, N. Franceschini, A.P. Reiner, M. Feitosa, J.J. Carr, P. Gordon-Larsen, M.K. Wojczynski, I.B. Borecki, PNPLA3 gene-by-visceral adipose tissue volume interaction and the pathogenesis of fatty liver disease: the NHLBI family heart study, Int. J. Obes. 37 (2013) 432–438.
[89] E.M. Giudice, A. Grandone, G. Cirillo, N. Santoro, A. Amato, C. Brienza, P. Savarese, P. Marzuillo, L. Perrone, The association of PNPLA3 variants with liver enzymes in childhood obesity is driven by the interaction with abdominal fat, PLoS One 6 (2011) e27933.
[90] J.N. Davis, K.A. Le, R.W. Walker, S. Vikman, D. Spruijt-Metz, M.J. Weigensberg, H. Allayee, M.I. Goran, Increased hepatic fat in overweight Hispanic youth influenced by interaction between genetic variation in PNPLA3 and high dietary carbohydrate and sugar consumption, Am. J. Clin. Nutr. 92 (2010) 1522–1527.
[91] C. Tian, R.P. Stokowski, D. Kershenobich, D.G. Ballinger, D.A. Hinds, Variant in PNPLA3 is associated with alcoholic liver disease, Nat. Genet. 42 (2010) 21–23.
[92] Z. Chamoun, F. Vacca, R.G. Parton, J. Gruenberg, PNPLA3/adiponutrin functions in lipid droplet formation, Biol. Cell 105 (2013) 219–233, under the auspices of the European Cell Biology Organization.
[93] J.Z. Li, Y. Huang, R. Karaman, P.T. Ivanova, H.A. Brown, T. Roddy, J. Castro-Perez, J.C. Cohen, H.H. Hobbs, Chronic overexpression of PNPLA3I148M in mouse liver causes hepatic steatosis, J. Clin. Invest. 122 (2012) 4130–4144.

[94] N. Chalasani, X. Guo, R. Loomba, M.O. Goodarzi, T. Haritunians, S. Kwon, J. Cui, K.D. Taylor, L. Wilson, O.W. Cummings, Y.D. Chen, J.I. Rotter, Genome-wide association study identifies variants associated with histologic features of nonalcoholic fatty liver disease, Gastroenterology 139 (2010) 1567–1576, 1576.e1–6.
[95] G.C. Ness, Z. Zhao, R.K. Keller, Effect of squalene synthase inhibition on the expression of hepatic cholesterol biosynthetic enzymes, LDL receptor, and cholesterol 7 alpha hydroxylase, Arch. Biochem. Biophys. 311 (1994) 277–285.
[96] I. Schechter, D.G. Conrad, I. Hart, R.C. Berger, T.L. McKenzie, J. Bleskan, D. Patterson, Localization of the squalene synthase gene (FDFT1) to human chromosome 8p22-p23.1, Genomics 20 (1994) 116–118.
[97] H. Okazaki, F. Tazoe, S. Okazaki, N. Isoo, K. Tsukamoto, M. Sekiya, N. Yahagi, Y. Iizuka, K. Ohashi, T. Kitamine, R. Tozawa, T. Inaba, H. Yagyu, M. Okazaki, H. Shimano, N. Shibata, H. Arai, R.Z. Nagai, T. Kadowaki, J. Osuga, S. Ishibashi, Increased cholesterol biosynthesis and hypercholesterolemia in mice overexpressing squalene synthase in the liver, J. Lipid Res. 47 (2006) 1950–1958.
[98] N. Santoro, C.K. Zhang, H. Zhao, A.J. Pakstis, G. Kim, R. Kursawe, D.J. Dykas, A.E. Bale, C. Giannini, B. Pierpont, M.M. Shaw, L. Groop, S. Caprio, Variant in the glucokinase regulatory protein (GCKR) gene is associated with fatty liver in obese children and adolescents, Hepatology 55 (2012) 781–789.
[99] N.L. Beer, N.D. Tribble, L.J. McCulloch, C. Roos, P.R. Johnson, M. Orho-Melander, A.L. Gloyn, The P446L variant in GCKR associated with fasting plasma glucose and triglyceride levels exerts its effect through increased glucokinase activity in liver, Hum. Mol. Genet. 18 (2009) 4081–4088.
[100] R. Weiss, Fat distribution and storage: how much, where, and how? Eur. J. Endocrinol. 157 (Suppl. 1) (2007) S39–S45, European Federation of Endocrine Societies.
[101] N. Santoro, R. Weiss, Metabolic syndrome in youth: current insights and novel serum biomarkers, Biomark. Med. 6 (2012) 719–727.
[102] S.E. Taksali, S. Caprio, J. Dziura, S. Dufour, A.M. Cali, T.R. Goodman, X. Papademetris, T.S. Burgert, B.M. Pierpont, M. Savoye, M. Shaw, A.A. Seyal, R. Weiss, High visceral and low abdominal subcutaneous fat stores in the obese adolescent: a determinant of an adverse metabolic phenotype, Diabetes 57 (2008) 367–371.
[103] R. Kursawe, M. Eszlinger, D. Narayan, T. Liu, M. Bazuine, A.M. Cali, E. D'Adamo, M. Shaw, B. Pierpont, G.I. Shulman, S.W. Cushman, A. Sherman, S. Caprio, Cellularity and adipogenic profile of the abdominal subcutaneous adipose tissue from obese adolescents: association with insulin resistance and hepatic steatosis, Diabetes 59 (2010) 2288–2296.
[104] C. Koutsari, M.D. Jensen, Thematic review series: patient-oriented research. Free fatty acid metabolism in human obesity, J. Lipid Res. 47 (2006) 1643–1650.
[105] A.H. Berg, P.E. Scherer, Adipose tissue, inflammation, and cardiovascular disease, Circ. Res. 96 (2005) 939–949.
[106] X.J. Xu, M.S. Gauthier, D.T. Hess, C.M. Apovian, J.M. Cacicedo, N. Gokce, M. Farb, R.J. Valentine, N.B. Ruderman, Insulin sensitive and resistant obesity in humans: AMPK activity, oxidative stress, and depot-specific changes in gene expression in adipose tissue, J. Lipid Res. 53 (2012) 792–801.
[107] G.H. Goossens, A. Bizzarri, N. Venteclef, Y. Essers, J.P. Cleutjens, E. Konings, J.W. Jocken, M. Cajlakovic, V. Ribitsch, K. Clement, E.E. Blaak, Increased adipose tissue oxygen tension in obese compared with lean men is accompanied by insulin resistance, impaired adipose tissue capillarization, and inflammation, Circulation 124 (2011) 67–76.
[108] M.P. Gillum, M.E. Kotas, D.M. Erion, R. Kursawe, P. Chatterjee, K.T. Nead, E.S. Muise, J.J. Hsiao, D.W. Frederick, S. Yonemitsu, A.S. Banks, L. Qiang,

S. Bhanot, J.M. Olefsky, D.D. Sears, S. Caprio, G.I. Shulman, SirT1 regulates adipose tissue inflammation, Diabetes 60 (2011) 3235–3245.
[109] O.T. Hardy, R.A. Perugini, S.M. Nicoloro, K. Gallagher-Dorval, V. Puri, J. Straubhaar, M.P. Czech, Body mass index-independent inflammation in omental adipose tissue associated with insulin resistance in morbid obesity, Surg. Obes. Relat. Dis. 7 (2011) 60–67.
[110] M.F. Gregor, L. Yang, E. Fabbrini, B.S. Mohammed, J.C. Eagon, G.S. Hotamisligil, S. Klein, Endoplasmic reticulum stress is reduced in tissues of obese subjects after weight loss, Diabetes 58 (2009) 693–700.
[111] E.K. Anderson, D.A. Gutierrez, A.H. Hasty, Adipose tissue recruitment of leukocytes, Curr. Opin. Lipidol. 21 (2010) 172–177.
[112] M. Pasarica, B. Gowronska-Kozak, D. Burk, I. Remedios, D. Hymel, J. Gimble, E. Ravussin, G.A. Bray, S.R. Smith, Adipose tissue collagen VI in obesity, J. Clin. Endocrinol. Metab. 94 (2009) 5155–5162.
[113] UK Prospective Diabetes Study (UKPDS) Group, Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34), Lancet 352 (1998) 854–865.
[114] V. Fonseca, Effect of thiazolidinediones on body weight in patients with diabetes mellitus, Am. J. Med. 115 (Suppl. 8A) (2003) 42S–48S.
[115] K.N. Frayn, Adipose tissue as a buffer for daily lipid flux, Diabetologia 45 (2002) 1201–1210.
[116] G.D. Kolovou, K.K. Anagnostopoulou, A.N. Pavlidis, K.D. Salpea, S.A. Iraklianou, K. Tsarpalis, D.S. Damaskos, A. Manolis, D.V. Cokkinos, Postprandial lipemia in men with metabolic syndrome, hypertensives and healthy subjects, Lipids Health Dis. 4 (2005) 21.
[117] D. Corella, L. Qi, E.S. Tai, M. Deurenberg-Yap, C.E. Tan, S.K. Chew, J.M. Ordovas, Perilipin gene variation determines higher susceptibility to insulin resistance in Asian women when consuming a high-saturated fat, low-carbohydrate diet, Diabetes Care 29 (2006) 1313–1319.
[118] M.D. Jensen, Adipose tissue and fatty acid metabolism in humans, J. R. Soc. Med. 95 (Suppl. 42) (2002) 3–7.
[119] S.A. Porter, J.M. Massaro, U. Hoffmann, R.S. Vasan, C.J. O'Donnel, C.S. Fox, Abdominal subcutaneous adipose tissue: a protective fat depot? Diabetes Care 32 (2009) 1068–1075.
[120] B.H. Goodpaster, F.L. Thaete, J.A. Simoneau, D.E. Kelley, Subcutaneous abdominal fat and thigh muscle composition predict insulin sensitivity independently of visceral fat, Diabetes 46 (1997) 1579–1585.
[121] N. Abate, A. Garg, R.M. Peshock, J. Stray-Gundersen, S.M. Grundy, Relationships of generalized and regional adiposity to insulin sensitivity in men, J. Clin. Invest. 96 (1995) 88–98.
[122] N. Abate, A. Garg, R.M. Peshock, J. Stray-Gundersen, B. Adams-Huet, S.M. Grundy, Relationship of generalized and regional adiposity to insulin sensitivity in men with NIDDM, Diabetes 45 (1996) 1684–1693.
[123] I. Ferreira, R.M. Henry, J.W. Twisk, W. van Mechelen, H.C. Kemper, C.D. Stehouwer, Amsterdam Growth and Health Longitudinal Study, The metabolic syndrome, cardiopulmonary fitness, and subcutaneous trunk fat as independent determinants of arterial stiffness: the Amsterdam Growth and Health Longitudinal Study, Arch. Intern. Med. 165 (2005) 875–882.
[124] U. Salmenniemi, E. Ruotsalainen, J. Pihlajamaki, I. Vauhkonen, S. Kainulainen, K. Punnonen, E. Vanninen, M. Laakso, Multiple abnormalities in glucose and energy metabolism and coordinated changes in levels of adiponectin, cytokines, and adhesion molecules in subjects with metabolic syndrome, Circulation 110 (2004) 3842–3848.

[125] D.B. Carr, K.M. Utzschneider, R.L. Hull, K. Kodama, B.M. Retzlaff, J.D. Brunzell, J.B. Shofer, B.E. Fish, R.H. Knopp, S.E. Kahn, Intra-abdominal fat is a major determinant of the National Cholesterol Education Program Adult Treatment Panel III criteria for the metabolic syndrome, Diabetes 53 (2004) 2087–2094.
[126] C.M. Apovian, S. Bigornia, M. Mott, M.R. Meyers, J. Ulloor, M. Gagua, M. McDonnell, D. Hess, L. Joseph, N. Gokce, Adipose macrophage infiltration is associated with insulin resistance and vascular endothelial dysfunction in obese subjects, Arterioscler. Thromb. Vasc. Biol. 28 (2008) 1654–1659.
[127] H. Kurosu, M. Choi, Y. Ogawa, A.S. Dickson, R. Goetz, A.V. Eliseenkova, M. Mohammadi, K.P. Rosenblatt, S.A. Kliewer, M. Kuro-o, Tissue-specific expression of betaKlotho and fibroblast growth factor (FGF) receptor isoforms determines metabolic activity of FGF19 and FGF21, J. Biol. Chem. 282 (2007) 26687–26695.
[128] J. Yie, R. Hecht, J. Patel, J. Stevens, W. Wang, N. Hawkins, S. Steavenson, S. Smith, D. Winters, S. Fisher, L. Cai, E. Belouski, C. Chen, M.L. Michaels, Y.S. Li, R. Lindberg, M. Wang, M. Veniant, J. Xu, FGF21 N- and C-termini play different roles in receptor interaction and activation, FEBS Lett. 583 (2009) 19–24.
[129] A. Kharitonenkov, J.D. Dunbar, H.A. Bina, S. Bright, J.S. Moyers, C. Zhang, L. Ding, R. Micanovic, S.F. Mehrbod, M.D. Knierman, J.E. Hale, T. Coskun, A.B. Shanafelt, FGF-21/FGF-21 receptor interaction and activation is determined by betaKlotho, J. Cell. Physiol. 215 (2008) 1–7.
[130] D.A. Sarruf, J.P. Thaler, G.J. Morton, J. German, J.D. Fischer, K. Ogimoto, M.W. Schwartz, Fibroblast growth factor 21 action in the brain increases energy expenditure and insulin sensitivity in obese rats, Diabetes 59 (2010) 1817–1824.
[131] C. Galman, T. Lundasen, A. Kharitonenkov, H.A. Bina, M. Eriksson, I. Hafstrom, M. Dahlin, P. Amark, B. Angelin, M. Rudling, The circulating metabolic regulator FGF21 is induced by prolonged fasting and PPARalpha activation in man, Cell Metab. 8 (2008) 169–174.
[132] T. Inagaki, P. Dutchak, G. Zhao, X. Ding, L. Gautron, V. Parameswara, Y. Li, R. Goetz, M. Mohammadi, V. Esser, J.K. Elmquist, R.D. Gerard, S.C. Burgess, R.E. Hammer, D.J. Mangelsdorf, S.A. Kliewer, Endocrine regulation of the fasting response by PPARalpha-mediated induction of fibroblast growth factor 21, Cell Metab. 5 (2007) 415–425.
[133] J. Sanchez, A. Palou, C. Pico, Response to carbohydrate and fat refeeding in the expression of genes involved in nutrient partitioning and metabolism: striking effects on fibroblast growth factor-21 induction, Endocrinology 150 (2009) 5341–5350.
[134] E.M. Domouzoglou, E. Maratos-Flier, Fibroblast growth factor 21 is a metabolic regulator that plays a role in the adaptation to ketosis, Am. J. Clin. Nutr. 93 (2011) 901S–905S.
[135] C. Giannini, A.E. Feldstein, N. Santoro, G. Kim, R. Kursawe, B. Pierpont, S. Caprio, Circulating levels of FGF-21 in obese youth: associations with liver fat content and markers of liver damage, J. Clin. Endocrinol. Metab. 98 (2013) 2993–3000.
[136] K.W. Williams, M.M. Scott, J.K. Elmquist, From observation to experimentation: leptin action in the mediobasal hypothalamus, Am. J. Clin. Nutr. 89 (2009) 985S–990S.
[137] D.M. Muoio, G. Lynis Dohm, Peripheral metabolic actions of leptin, Best Pract. Res. Clin. Endocrinol. Metab. 16 (2002) 653–666.
[138] R.S. Ahima, D. Prabakaran, C. Mantzoros, D. Qu, B. Lowell, E. Maratos-Flier, J.S. Flier, Role of leptin in the neuroendocrine response to fasting, Nature 382 (1996) 250–252.
[139] J.S. Flier, Clinical review 94: what's in a name? In search of leptin's physiologic role, J. Clin. Endocrinol. Metab. 83 (1998) 1407–1413.
[140] M. Halle, P.B. Persson, Role of leptin and leptin receptor in inflammation, Am. J. Physiol. Endocrinol. Metab. 284 (2003) R760–R762.

[141] S. Margetic, C. Gazzola, G.G. Pegg, R.A. Hill, Leptin: a review of its peripheral actions and interactions, Int. J. Obes. Relat. Metab. Disord. 26 (2002) 1407–1433.
[142] T. Tsuchiya, H. Shimizu, T. Horie, M. Mori, Expression of leptin receptor in lung: leptin as a growth factor, Eur. J. Pharmacol. 365 (1999) 273–279.
[143] M. Artwohl, M. Roden, T. Holzenbein, A. Freudenthaler, W. Waldhausl, S.M. Baumgartner-Parzer, Modulation by leptin of proliferation and apoptosis in vascular endothelial cells, Int. J. Obes. Relat. Metab. Disord. 26 (2002) 577–580.
[144] M. Koda, L. Kanczuga-Koda, M. Sulkowska, E. Surmacz, S. Sulkowski, Relationships between hypoxia markers and the leptin system, estrogen receptors in human primary and metastatic breast cancer: effects of preoperative chemotherapy, BMC Cancer 10 (2010) 320.
[145] C. Bjorbaek, B.B. Kahn, Leptin signaling in the central nervous system and the periphery, Recent Prog. Horm. Res. 59 (2004) 305–331.
[146] C. Dal Farra, N. Zsurger, J.P. Vincent, A. Cupo, Binding of a pure 125I-monoiodoleptin analog to mouse tissues: a developmental study, Peptides 21 (2000) 577–587.
[147] M. Ciccone, R. Vettor, N. Pannacciulli, A. Minenna, M. Bellacicco, P. Rizzon, R. Giorgino, G. De Pergola, Plasma leptin is independently associated with the intima-media thickness of the common carotid artery, Int. J. Obes. Relat. Metab. Disord. 25 (2001) 805–810.
[148] S.B. Heymsfield, A.S. Greenberg, K. Fujioka, R.M. Dixon, R. Kushner, T. Hunt, J.A. Lubina, J. Patane, B. Self, P. Hunt, M. McCamish, Recombinant leptin for weight loss in obese and lean adults: a randomized, controlled, dose-escalation trial, JAMA 282 (1999) 1568–1575.
[149] Y. Arita, S. Kihara, N. Ouchi, M. Takahashi, K. Maeda, J. Miyagawa, K. Hotta, I. Shimomura, T. Nakamura, K. Miyaoka, H. Kuriyama, M. Nishida, S. Yamashita, K. Okubo, K. Matsubara, M. Muraguchi, Y. Ohmoto, T. Funahashi, Y. Matsuzawa, Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. 1999, Biochem. Biophys. Res. Commun. 425 (2012) 560–564.
[150] O. Tschritter, A. Fritsche, C. Thamer, M. Haap, F. Shirkavand, S. Rahe, H. Staiger, E. Maerker, H. Haring, M. Stumvoll, Plasma adiponectin concentrations predict insulin sensitivity of both glucose and lipid metabolism, Diabetes 52 (2003) 239–243.
[151] C. Weyer, T. Funahashi, S. Tanaka, K. Hotta, Y. Matsuzawa, R.E. Pratley, P.A. Tataranni, Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia, J. Clin. Endocrinol. Metab. 86 (2001) 1930–1935.
[152] M. Daimon, T. Oizumi, T. Saitoh, W. Kameda, A. Hirata, H. Yamaguchi, H. Ohnuma, M. Igarashi, M. Tominaga, T. Kato, Decreased serum levels of adiponectin are a risk factor for the progression to type 2 diabetes in the Japanese population: the Funagata study, Diabetes Care 26 (2003) 2015–2020.
[153] C. Snehalatha, B. Mukesh, M. Simon, V. Viswanathan, S.M. Haffner, A. Ramachandran, Plasma adiponectin is an independent predictor of type 2 diabetes in Asian Indians, Diabetes Care 26 (2003) 3226–3229.
[154] J. Spranger, A. Kroke, M. Mohlig, M.M. Bergmann, M. Ristow, H. Boeing, A.F. Pfeiffer, Adiponectin and protection against type 2 diabetes mellitus, Lancet 361 (2003) 226–228.
[155] R.S. Lindsay, T. Funahashi, R.L. Hanson, Y. Matsuzawa, S. Tanaka, P.A. Tataranni, W.C. Knowler, J. Krakoff, Adiponectin and development of type 2 diabetes in the Pima Indian population, Lancet 360 (2002) 57–58.
[156] T.P. Combs, A.H. Berg, S. Obici, P.E. Scherer, L. Rossetti, Endogenous glucose production is inhibited by the adipose-derived protein Acrp30, J. Clin. Invest. 108 (2001) 1875–1881.

[157] N. Ouchi, S. Kihara, Y. Arita, M. Nishida, A. Matsuyama, Y. Okamoto, M. Ishigami, H. Kuriyama, K. Kishida, H. Nishizawa, K. Hotta, M. Muraguchi, Y. Ohmoto, S. Yamashita, T. Funahashi, Y. Matsuzawa, Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class A scavenger receptor expression in human monocyte-derived macrophages, Circulation 103 (2001) 1057–1063.
[158] T. Yamauchi, J. Kamon, H. Waki, Y. Terauchi, N. Kubota, K. Hara, Y. Mori, T. Ide, K. Murakami, N. Tsuboyama-Kasaoka, O. Ezaki, Y. Akanuma, O. Gavrilova, C. Vinson, M.L. Reitman, H. Kagechika, K. Shudo, M. Yoda, Y. Nakano, K. Tobe, R. Nagai, S. Kimura, M. Tomita, P. Froguel, T. Kadowaki, The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity, Nat. Med. 7 (2001) 941–946.
[159] T. Yamauchi, J. Kamon, Y. Minokoshi, Y. Ito, H. Waki, S. Uchida, S. Yamashita, M. Noda, S. Kita, K. Ueki, K. Eto, Y. Akanuma, P. Froguel, F. Foufelle, P. Ferre, D. Carling, S. Kimura, R. Nagai, B.B. Kahn, T. Kadowaki, Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase, Nat. Med. 8 (2002) 1288–1295.
[160] X. Wu, H. Motoshima, K. Mahadev, T.J. Stalker, R. Scalia, B.J. Goldstein, Involvement of AMP-activated protein kinase in glucose uptake stimulated by the globular domain of adiponectin in primary rat adipocytes, Diabetes 52 (2003) 1355–1363.
[161] A. Tsuchida, T. Yamauchi, S. Takekawa, Y. Hada, Y. Ito, T. Maki, T. Kadowaki, Peroxisome proliferator-activated receptor (PPAR)alpha activation increases adiponectin receptors and reduces obesity-related inflammation in adipose tissue: comparison of activation of PPARalpha, PPARgamma, and their combination, Diabetes 54 (2005) 3358–3370.
[162] T. Yamauchi, J. Kamon, Y. Ito, A. Tsuchida, T. Yokomizo, S. Kita, T. Sugiyama, M. Miyagishi, K. Hara, M. Tsunoda, K. Murakami, T. Ohteki, S. Uchida, S. Takekawa, H. Waki, N.H. Tsuno, Y. Shibata, Y. Terauchi, P. Froguel, K. Tobe, S. Koyasu, K. Taira, T. Kitamura, T. Shimizu, R. Nagai, T. Kadowaki, Cloning of adiponectin receptors that mediate antidiabetic metabolic effects, Nature 423 (2003) 762–769.
[163] N. Kubota, W. Yano, T. Kubota, T. Yamauchi, S. Itoh, H. Kumagai, H. Kozono, I. Takamoto, S. Okamoto, T. Shiuchi, R. Suzuki, H. Satoh, A. Tsuchida, M. Moroi, K. Sugi, T. Noda, H. Ebinuma, Y. Ueta, T. Kondo, E. Araki, O. Ezaki, R. Nagai, K. Tobe, Y. Terauchi, K. Ueki, Y. Minokoshi, T. Kadowaki, Adiponectin stimulates AMP-activated protein kinase in the hypothalamus and increases food intake, Cell Metab. 6 (2007) 55–68.
[164] J.A. Morrison, C.J. Glueck, S. Daniels, P. Wang, D. Stroop, Paradoxically high adiponectin in obese 16-year-old girls protects against appearance of the metabolic syndrome and its components seven years later, J. Pediatr. 158 (2011) 208.e1–214.e1.
[165] S. Lee, F. Bacha, N. Gungor, S.A. Arslanian, Racial differences in adiponectin in youth: relationship to visceral fat and insulin sensitivity, Diabetes Care 29 (2006) 51–56.
[166] I. Kynde, B.L. Heitmann, I.C. Bygbjerg, L.B. Andersen, J.W. Helge, Hypoadiponectinemia in overweight children contributes to a negative metabolic risk profile 6 years later, Metab. Clin. Exp. 58 (2009) 1817–1824.
[167] E.S. Ford, C. Li, S. Cook, H.K. Choi, Serum concentrations of uric acid and the metabolic syndrome among US children and adolescents, Circulation 115 (2007) 2526–2532.
[168] M.C. Ernst, C.J. Sinal, Chemerin: at the crossroads of inflammation and obesity, Trends Endocrinol. Metab. 21 (2010) 660–667.
[169] K. Bozaoglu, D. Segal, K.A. Shields, N. Cummings, J.E. Curran, A.G. Comuzzie, M.C. Mahaney, D.L. Rainwater, J.L. VandeBerg, J.W. MacCluer, G. Collier,

J. Blangero, K. Walder, J.B. Jowett, Chemerin is associated with metabolic syndrome phenotypes in a Mexican-American population, J. Clin. Endocrinol. Metab. 94 (2009) 3085–3088.
[170] H. Sell, A. Divoux, C. Poitou, A. Basdevant, J.L. Bouillot, P. Bedossa, J. Tordjman, J. Eckel, K. Clement, Chemerin correlates with markers for fatty liver in morbidly obese patients and strongly decreases after weight loss induced by bariatric surgery, J. Clin. Endocrinol. Metab. 95 (2010) 2892–2896.
[171] A.A. Bremer, I. Jialal, Adipose tissue dysfunction in nascent metabolic syndrome, J. Obes. 2013 (2013) 393192.
[172] A.A. Bremer, S. Devaraj, A. Afify, I. Jialal, Adipose tissue dysregulation in patients with metabolic syndrome, J. Clin. Endocrinol. Metab. 96 (2011) E1782–E1788.
[173] I. Jialal, S. Devaraj, B. Adams-Huet, X. Chen, H. Kaur, Increased cellular and circulating biomarkers of oxidative stress in nascent metabolic syndrome, J. Clin. Endocrinol. Metab. 97 (2012) E1844–E1850.
[174] D. Stejskal, M. Karpisek, Z. Hanulova, M. Svestak, Chemerin is an independent marker of the metabolic syndrome in a Caucasian population—a pilot study, Biomed. Pap. Med. Fac. Univ. Palacky Olomouc Czech. Repub. 152 (2008) 217–221.
[175] S.H. Chu, M.K. Lee, K.Y. Ahn, J.A. Im, M.S. Park, D.C. Lee, J.Y. Jeon, J.W. Lee, Chemerin and adiponectin contribute reciprocally to metabolic syndrome, PLoS One 7 (2012) e34710.
[176] B. Dong, W. Ji, Y. Zhang, Elevated serum chemerin levels are associated with the presence of coronary artery disease in patients with metabolic syndrome, Intern. Med. 50 (2011) 1093–1097.
[177] J. Weigert, M. Neumeier, J. Wanninger, M. Filarsky, S. Bauer, R. Wiest, S. Farkas, M.N. Scherer, A. Schaffler, C. Aslanidis, J. Scholmerich, C. Buechler, Systemic chemerin is related to inflammation rather than obesity in type 2 diabetes, Clin. Endocrinol. 72 (2010) 342–348.
[178] R.Z. Yang, M.J. Lee, H. Hu, J. Pray, H.B. Wu, B.C. Hansen, A.R. Shuldiner, S.K. Fried, J.C. McLenithan, D.W. Gong, Identification of omentin as a novel depot-specific adipokine in human adipose tissue: possible role in modulating insulin action, Am. J. Physiol. Endocrinol. Metab. 290 (2006) E1253–E1261.
[179] B.K. Tan, S. Pua, F. Syed, K.C. Lewandowski, J.P. O'Hare, H.S. Randeva, Decreased plasma omentin-1 levels in type 1 diabetes mellitus, Diabet. Med. 25 (2008) 1254–1255.
[180] C. Jaikanth, P. Gurumurthy, K.M. Cherian, T. Indhumathi, Emergence of omentin as a pleiotropic adipocytokine, Exp. Clin. Endocrinol. Diabetes 121 (2013) 377–383.
[181] J.N. Fain, P.S. Cheema, S.W. Bahouth, M. Lloyd Hiler, Resistin release by human adipose tissue explants in primary culture, Biochem. Biophys. Res. Commun. 300 (2003) 674–678.
[182] L. Patel, A.C. Buckels, I.J. Kinghorn, P.R. Murdock, J.D. Holbrook, C. Plumpton, C.H. Macphee, S.A. Smith, Resistin is expressed in human macrophages and directly regulated by PPAR gamma activators, Biochem. Biophys. Res. Commun. 300 (2003) 472–476.
[183] M. Gerber, A. Boettner, B. Seidel, A. Lammert, J. Bar, E. Schuster, J. Thiery, W. Kiess, J. Kratzsch, Serum resistin levels of obese and lean children and adolescents: biochemical analysis and clinical relevance, J. Clin. Endocrinol. Metab. 90 (2005) 4503–4509.
[184] D.B. Savage, C.P. Sewter, E.S. Klenk, D.G. Segal, A. Vidal-Puig, R.V. Considine, S. O'Rahilly, Resistin/Fizz3 expression in relation to obesity and peroxisome proliferator-activated receptor-gamma action in humans, Diabetes 50 (2001) 2199–2202.
[185] P.G. McTernan, F.M. Fisher, G. Valsamakis, R. Chetty, A. Harte, C.L. McTernan, P.M. Clark, S.A. Smith, A.H. Barnett, S. Kumar, Resistin and type 2 diabetes: regulation of resistin expression by insulin and rosiglitazone and the effects of recombinant

resistin on lipid and glucose metabolism in human differentiated adipocytes, J. Clin. Endocrinol. Metab. 88 (2003) 6098–6106.
[186] M.P. Reilly, M. Lehrke, M.L. Wolfe, A. Rohatgi, M.A. Lazar, D.J. Rader, Resistin is an inflammatory marker of atherosclerosis in humans, Circulation 111 (2005) 932–939.
[187] M.F. Hivert, L.M. Sullivan, C.S. Fox, D.M. Nathan, R.B. D'Agostino Sr., P.W. Wilson, J.B. Meigs, Associations of adiponectin, resistin, and tumor necrosis factor-alpha with insulin resistance, J. Clin. Endocrinol. Metab. 93 (2008) 3165–3172.
[188] G.D. Norata, M. Ongari, K. Garlaschelli, S. Raselli, L. Grigore, A.L. Catapano, Plasma resistin levels correlate with determinants of the metabolic syndrome, Eur. J. Endocrinol. 156 (2007) 279–284.
[189] T. Pischon, C.M. Bamberger, J. Kratzsch, B.C. Zyriax, P. Algenstaedt, H. Boeing, E. Windler, Association of plasma resistin levels with coronary heart disease in women, Obes. Res. 13 (2005) 1764–1771.
[190] D.S. Frankel, R.S. Vasan, R.B. D'Agostino Sr., E.J. Benjamin, D. Levy, T.J. Wang, J.B. Meigs, Resistin, adiponectin, and risk of heart failure the Framingham offspring study, J. Am. Coll. Cardiol. 53 (2009) 754–762.
[191] S. Lim, B.K. Koo, S.W. Cho, S. Kihara, T. Funahashi, Y.M. Cho, S.Y. Kim, H.K. Lee, I. Shimomura, K.S. Park, Association of adiponectin and resistin with cardiovascular events in Korean patients with type 2 diabetes: the Korean atherosclerosis study (KAS): a 42-month prospective study, Atherosclerosis 196 (2008) 398–404.
[192] S. Yaturu, R.P. Daberry, J. Rains, S. Jain, Resistin and adiponectin levels in subjects with coronary artery disease and type 2 diabetes, Cytokine 34 (2006) 219–223.
[193] M. Bokarewa, I. Nagaev, L. Dahlberg, U. Smith, A. Tarkowski, Resistin, an adipokine with potent proinflammatory properties, J. Immunol. 174 (2005) 5789–5795.
[194] P. Calabro, I. Samudio, J.T. Willerson, E.T. Yeh, Resistin promotes smooth muscle cell proliferation through activation of extracellular signal-regulated kinase 1/2 and phosphatidylinositol 3-kinase pathways, Circulation 110 (2004) 3335–3340.
[195] A. Garten, S. Petzold, A. Korner, S. Imai, W. Kiess, Nampt: linking NAD biology, metabolism and cancer, Trends Endocrinol. Metab. 20 (2009) 130–138.
[196] A. Garten, S. Petzold, A. Barnikol-Oettler, A. Korner, W.E. Thasler, J. Kratzsch, W. Kiess, R. Gebhardt, Nicotinamide phosphoribosyltransferase (NAMPT/PBEF/visfatin) is constitutively released from human hepatocytes, Biochem. Biophys. Res. Commun. 391 (2010) 376–381.
[197] S.R. Costford, S. Bajpeyi, M. Pasarica, D.C. Albarado, S.C. Thomas, H. Xie, T.S. Church, S.A. Jubrias, K.E. Conley, S.R. Smith, Skeletal muscle NAMPT is induced by exercise in humans, Am. J. Physiol. Endocrinol. Metab. 298 (2010) E117–126.
[198] S.M. Krzysik-Walker, O.M. Ocon-Grove, S.R. Maddineni, G.L. Hendricks 3rd., R. Ramachandran, Is visfatin an adipokine or myokine? Evidence for greater visfatin expression in skeletal muscle than visceral fat in chickens, Endocrinology 149 (2008) 1543–1550.
[199] J.R. Revollo, A. Korner, K.F. Mills, A. Satoh, T. Wang, A. Garten, B. Dasgupta, Y. Sasaki, C. Wolberger, R.R. Townsend, J. Milbrandt, W. Kiess, S. Imai, Nampt/PBEF/Visfatin regulates insulin secretion in beta cells as a systemic NAD biosynthetic enzyme, Cell Metab. 6 (2007) 363–375.
[200] A.R. Moschen, A. Kaser, B. Enrich, B. Mosheimer, M. Theurl, H. Niederegger, H. Tilg, Visfatin, an adipocytokine with proinflammatory and immunomodulating properties, J. Immunol. 178 (2007) 1748–1758.
[201] J. Berndt, N. Kloting, S. Kralisch, P. Kovacs, M. Fasshauer, M.R. Schon, M. Stumvoll, M. Bluher, Plasma visfatin concentrations and fat depot-specific mRNA expression in humans, Diabetes 54 (2005) 2911–2916.

[202] A. Jaleel, B. Aheed, S. Jaleel, R. Majeed, A. Zuberi, S. Khan, B. Ahmed, F. Shoukat, H. Hashim, Association of adipokines with obesity in children and adolescents, Biomark. Med. 7 (2013) 731–735.
[203] D.G. Haider, K. Schindler, G. Schaller, G. Prager, M. Wolzt, B. Ludvik, Increased plasma visfatin concentrations in morbidly obese subjects are reduced after gastric banding, J. Clin. Endocrinol. Metab. 91 (2006) 1578–1581.
[204] Y.S. Kang, H.K. Song, M.H. Lee, G.J. Ko, D.R. Cha, Plasma concentration of visfatin is a new surrogate marker of systemic inflammation in type 2 diabetic patients, Diabetes Res. Clin. Pract. 89 (2010) 141–149.
[205] C. Grunfeld, Leptin and the immunosuppression of malnutrition, J. Clin. Endocrinol. Metab. 87 (2002) 3038–3039.
[206] E.D. Abel, O. Peroni, J.K. Kim, Y.B. Kim, O. Boss, E. Hadro, T. Minnemann, G.I. Shulman, B.B. Kahn, Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver, Nature 409 (2001) 729–733.
[207] Q. Yang, T.E. Graham, N. Mody, F. Preitner, O.D. Peroni, J.M. Zabolotny, K. Kotani, L. Quadro, B.B. Kahn, Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes, Nature 436 (2005) 356–362.
[208] T.E. Graham, Q. Yang, M. Bluher, A. Hammarstedt, T.P. Ciaraldi, R.R. Henry, C.J. Wason, A. Oberbach, P.A. Jansson, U. Smith, B.B. Kahn, Retinol-binding protein 4 and insulin resistance in lean, obese, and diabetic subjects, N. Engl. J. Med. 354 (2006) 2552–2563.
[209] A. Yao-Borengasser, V. Varma, A.M. Bodles, N. Rasouli, B. Phanavanh, M.J. Lee, T. Starks, L.M. Kern, H.J. Spencer 3rd., A.A. Rashidi, R.E. McGehee Jr., S.K. Fried, P.A. Kern, Retinol binding protein 4 expression in humans: relationship to insulin resistance, inflammation, and response to pioglitazone, J. Clin. Endocrinol. Metab. 92 (2007) 2590–2597.
[210] P.G. McTernan, S. Kumar, Editorial: retinol binding protein 4 and pathogenesis of diabetes, J. Clin. Endocrinol. Metab. 92 (2007) 2430–2432.
[211] D. Friebe, M. Neef, S. Erbs, K. Dittrich, J. Kratzsch, P. Kovacs, M. Bluher, W. Kiess, A. Korner, Retinol binding protein 4 (RBP4) is primarily associated with adipose tissue mass in children, Int. J. Pediatr. Obes. 6 (2011) e345–e352.
[212] E. Goodman, T.E. Graham, L.M. Dolan, S.R. Daniels, E.R. Goodman, B.B. Kahn, The relationship of retinol binding protein 4 to changes in insulin resistance and cardiometabolic risk in overweight black adolescents, J. Pediatr. 154 (2009) 67. e1–73.e1.
[213] S. Gavi, S. Qurashi, M.M. Melendez, D.C. Mynarcik, M.A. McNurlan, M.C. Gelato, Plasma retinol-binding protein-4 concentrations are elevated in human subjects with impaired glucose tolerance and type 2 diabetes: response to Cho et al., Diabetes Care 30 (2007) e7, author reply e8.
[214] Y.M. Cho, B.S. Youn, H. Lee, N. Lee, S.S. Min, S.H. Kwak, H.K. Lee, K.S. Park, Plasma retinol-binding protein-4 concentrations are elevated in human subjects with impaired glucose tolerance and type 2 diabetes, Diabetes Care 29 (2006) 2457–2461.
[215] W. Jia, H. Wu, Y. Bao, C. Wang, J. Lu, J. Zhu, K. Xiang, Association of serum retinol-binding protein 4 and visceral adiposity in Chinese subjects with and without type 2 diabetes, J. Clin. Endocrinol. Metab. 92 (2007) 3224–3229.
[216] K.R. Kelly, S.R. Kashyap, V.B. O'Leary, J. Major, P.R. Schauer, J.P. Kirwan, Retinol-binding protein 4 (RBP4) protein expression is increased in omental adipose tissue of severely obese patients, Obesity (Silver Spring) 18 (2010) 663–666.
[217] N. Kloting, T.E. Graham, J. Berndt, S. Kralisch, P. Kovacs, C.J. Wason, M. Fasshauer, M.R. Schon, M. Stumvoll, M. Bluher, B.B. Kahn, Serum retinol-binding protein is more highly expressed in visceral than in subcutaneous adipose tissue and is a marker of intra-abdominal fat mass, Cell Metab. 6 (2007) 79–87.

[218] A.K. Nair, D. Sugunan, H. Kumar, G. Anilkumar, Case–control analysis of SNPs in GLUT4, RBP4 and STRA6: association of SNPs in STRA6 with type 2 diabetes in a South Indian population, PLoS One 5 (2010) e11444.
[219] S. Makino, M. Fujiwara, K. Suzukawa, H. Handa, T. Fujie, Y. Ohtaka, Y. Komatsu, Y. Aoki, H. Maruyama, Y. Terada, K. Hashimoto, T. Sugimoto, Visceral obesity is associated with the metabolic syndrome and elevated plasma retinol binding protein-4 level in obstructive sleep apnea syndrome, Horm. Metab. Res. 41 (2009) 221–226.
[220] Y. Wu, H. Li, R.J. Loos, Q. Qi, F.B. Hu, Y. Liu, X. Lin, RBP4 variants are significantly associated with plasma RBP4 levels and hypertriglyceridemia risk in Chinese Hans, J. Lipid Res. 50 (2009) 1479–1486.
[221] T. Bobbert, J. Raila, F. Schwarz, K. Mai, A. Henze, A.F. Pfeiffer, F.J. Schweigert, J. Spranger, Relation between retinol, retinol-binding protein 4, transthyretin and carotid intima media thickness, Atherosclerosis 213 (2010) 549–551.
[222] I. Aeberli, R. Biebinger, R. Lehmann, D. L'Allemand, G.A. Spinas, M.B. Zimmermann, Serum retinol-binding protein 4 concentration and its ratio to serum retinol are associated with obesity and metabolic syndrome components in children, J. Clin. Endocrinol. Metab. 92 (2007) 4359–4365.
[223] N. Santoro, L. Perrone, G. Cirillo, C. Brienza, A. Grandone, N. Cresta, E. Miraglia del Giudice, Variations of retinol binding protein 4 levels are not associated with changes in insulin resistance during puberty, J. Endocrinol. Investig. 32 (2009) 411–414.
[224] M. von Eynatten, P.M. Lepper, D. Liu, K. Lang, M. Baumann, P.P. Nawroth, A. Bierhaus, K.A. Dugi, U. Heemann, B. Allolio, P.M. Humpert, Retinol-binding protein 4 is associated with components of the metabolic syndrome, but not with insulin resistance, in men with type 2 diabetes or coronary artery disease, Diabetologia 50 (2007) 1930–1937.
[225] A.O. Chavez, D.K. Coletta, S. Kamath, D.T. Cromack, A. Monroy, F. Folli, R.A. DeFronzo, D. Tripathy, Retinol-binding protein 4 is associated with impaired glucose tolerance but not with whole body or hepatic insulin resistance in Mexican Americans, Am. J. Physiol. Endocrinol. Metab. 296 (2009) E758–E764.
[226] A.T. Kraja, M.A. Province, D. Arnett, L. Wagenknecht, W. Tang, P.N. Hopkins, L. Djousse, I.B. Borecki, Do inflammation and procoagulation biomarkers contribute to the metabolic syndrome cluster? Nutr. Metab. 4 (2007) 28.
[227] A.J. Hanley, A. Festa, R.B. D'Agostino Jr., L.E. Wagenknecht, P.J. Savage, R.P. Tracy, M.F. Saad, S.M. Haffner, Metabolic and inflammation variable clusters and prediction of type 2 diabetes: factor analysis using directly measured insulin sensitivity, Diabetes 53 (2004) 1773–1781.
[228] W. Tang, M.B. Miller, S.S. Rich, K.E. North, J.S. Pankow, I.B. Borecki, R.H. Myers, P.N. Hopkins, M. Leppert, D.K. Arnett, National Heart, Lung, and Blood Institute Family Heart Study, Linkage analysis of a composite factor for the multiple metabolic syndrome: the National Heart, Lung, and Blood Institute Family Heart Study, Diabetes 52 (2003) 2840–2847.
[229] P.A. Sakkinen, P. Wahl, M. Cushman, M.R. Lewis, R.P. Tracy, Clustering of procoagulation, inflammation, and fibrinolysis variables with metabolic factors in insulin resistance syndrome, Am. J. Epidemiol. 152 (2000) 897–907.
[230] Y. Aso, S. Wakabayashi, R. Yamamoto, R. Matsutomo, K. Takebayashi, T. Inukai, Metabolic syndrome accompanied by hypercholesterolemia is strongly associated with proinflammatory state and impairment of fibrinolysis in patients with type 2 diabetes: synergistic effects of plasminogen activator inhibitor-1 and thrombin-activatable fibrinolysis inhibitor, Diabetes Care 28 (2005) 2211–2216.
[231] R.E. Gimeno, L.D. Klaman, Adipose tissue as an active endocrine organ: recent advances, Curr. Opin. Pharmacol. 5 (2005) 122–128.

[232] X. Liang, T. Kanjanabuch, S.L. Mao, C.M. Hao, Y.W. Tang, P.J. Declerck, A.H. Hasty, D.H. Wasserman, A.B. Fogo, L.J. Ma, Plasminogen activator inhibitor-1 modulates adipocyte differentiation, Am. J. Physiol. Endocrinol. Metab. 290 (2006) E103–E113.
[233] W.P. Fay, A.C. Parker, L.R. Condrey, A.D. Shapiro, Human plasminogen activator inhibitor-1 (PAI-1) deficiency: characterization of a large kindred with a null mutation in the PAI-1 gene, Blood 90 (1997) 204–208.
[234] M. Swiatkowska, J. Szemraj, C.S. Cierniewski, Induction of PAI-1 expression by tumor necrosis factor alpha in endothelial cells is mediated by its responsive element located in the 4G/5G site, FEBS J. 272 (2005) 5821–5831.
[235] L.A. Erickson, G.J. Fici, J.E. Lund, T.P. Boyle, H.G. Polites, K.R. Marotti, Development of venous occlusions in mice transgenic for the plasminogen activator inhibitor-1 gene, Nature 346 (1990) 74–76.
[236] M. Eren, C.A. Painter, J.B. Atkinson, P.J. Declerck, D.E. Vaughan, Age-dependent spontaneous coronary arterial thrombosis in transgenic mice that express a stable form of human plasminogen activator inhibitor-1, Circulation 106 (2002) 491–496.
[237] A. Smith, C. Patterson, J. Yarnell, A. Rumley, Y. Ben-Shlomo, G. Lowe, Which hemostatic markers add to the predictive value of conventional risk factors for coronary heart disease and ischemic stroke? The Caerphilly Study, Circulation 112 (2005) 3080–3087.
[238] I. Mertens, A. Verrijken, J.J. Michiels, M. Van der Planken, J.B. Ruige, L.F. Van Gaal, Among inflammation and coagulation markers, PAI-1 is a true component of the metabolic syndrome, Int. J. Obes. 30 (2006) 1308–1314.
[239] P. Trayhurn, I.S. Wood, Signalling role of adipose tissue: adipokines and inflammation in obesity, Biochem. Soc. Trans. 33 (2005) 1078–1081.
[240] T. Skurk, H. Hauner, Obesity and impaired fibrinolysis: role of adipose production of plasminogen activator inhibitor-1, Int. J. Obes. Relat. Metab. Disord. 28 (2004) 1357–1364.
[241] S. Crowe, L.E. Wu, C. Economou, S.M. Turpin, M. Matzaris, K.L. Hoehn, A.L. Hevener, D.E. James, E.J. Duh, M.J. Watt, Pigment epithelium-derived factor contributes to insulin resistance in obesity, Cell Metab. 10 (2009) 40–47.
[242] S. Famulla, D. Lamers, S. Hartwig, W. Passlack, A. Horrighs, A. Cramer, S. Lehr, H. Sell, J. Eckel, Pigment epithelium-derived factor (PEDF) is one of the most abundant proteins secreted by human adipocytes and induces insulin resistance and inflammatory signaling in muscle and fat cells, Int. J. Obes. 35 (2011) 762–772.
[243] E. Banumathi, S. Sheikpranbabu, R. Haribalaganesh, S. Gurunathan, PEDF prevents reactive oxygen species generation and retinal endothelial cell damage at high glucose levels, Exp. Eye Res. 90 (2010) 89–96.
[244] S.X. Zhang, J.J. Wang, A. Dashti, K. Wilson, M.H. Zou, L. Szweda, J.X. Ma, T.J. Lyons, Pigment epithelium-derived factor mitigates inflammation and oxidative stress in retinal pericytes exposed to oxidized low-density lipoprotein, J. Mol. Endocrinol. 41 (2008) 135–143.
[245] H. Umei, S.I. Yamagishi, T. Imaizumi, Positive association of serum levels of pigment epithelium-derived factor with high-sensitivity C-reactive protein in apparently healthy unmedicated subjects, J. Int. Med. Res. 38 (2010) 443–448.
[246] D.W. Dawson, O.V. Volpert, P. Gillis, S.E. Crawford, H. Xu, W. Benedict, N.P. Bouck, Pigment epithelium-derived factor: a potent inhibitor of angiogenesis, Science 285 (1999) 245–248.
[247] C. Chen, A.W. Tso, L.S. Law, B.M. Cheung, K.L. Ong, N.M. Wat, E.D. Janus, A. Xu, K.S. Lam, Plasma level of pigment epithelium-derived factor is independently associated with the development of the metabolic syndrome in Chinese men: a 10-year prospective study, J. Clin. Endocrinol. Metab. 95 (2010) 5074–5081.

[248] D. Stejskal, M. Karpisek, M. Svestak, P. Hejduk, L. Sporova, H. Kotolova, Pigment epithelium-derived factor as a new marker of metabolic syndrome in Caucasian population, J. Clin. Lab. Anal. 24 (2010) 17–19.
[249] A.K. Gattu, A.L. Birkenfeld, F. Jornayvaz, J. Dziura, F. Li, S.E. Crawford, X. Chu, C.D. Still, G.S. Gerhard, C. Chung, V. Samuel, Insulin resistance is associated with elevated serum pigment epithelium-derived factor (PEDF) levels in morbidly obese patients, Acta Diabetol. 49 (Suppl. 1) (2012) S161–S169.
[250] L. Notari, V. Baladron, J.D. Aroca-Aguilar, N. Balko, R. Heredia, C. Meyer, P.M. Notario, S. Saravanamuthu, M.L. Nueda, F. Sanchez-Sanchez, J. Escribano, J. Laborda, S.P. Becerra, Identification of a lipase-linked cell membrane receptor for pigment epithelium-derived factor, J. Biol. Chem. 281 (2006) 38022–38037.
[251] L. Notari, N. Arakaki, D. Mueller, S. Meier, J. Amaral, S.P. Becerra, Pigment epithelium-derived factor binds to cell-surface F(1)-ATP synthase, FEBS J. 277 (2010) 2192–2205.
[252] J.M. Ordovas, D. Corella, Metabolic syndrome pathophysiology: the role of adipose tissue, Kidney Int. 74 (Suppl.) (2008) S10–S14.
[253] S. Gandotra, C. Le Dour, W. Bottomley, P. Cervera, P. Giral, Y. Reznik, G. Charpentier, M. Auclair, M. Delepine, I. Barroso, R.K. Semple, M. Lathrop, O. Lascols, J. Capeau, S. O'Rahilly, J. Magre, D.B. Savage, C. Vigouroux, Perilipin deficiency and autosomal dominant partial lipodystrophy, N. Engl. J. Med. 364 (2011) 740–748.
[254] D.L. Brasaemle, Thematic review series: adipocyte biology. The perilipin family of structural lipid droplet proteins: stabilization of lipid droplets and control of lipolysis, J. Lipid Res. 48 (2007) 2547–2559.
[255] M. Lafontan, D. Langin, Lipolysis and lipid mobilization in human adipose tissue, Prog. Lipid Res. 48 (2009) 275–297.
[256] A.W. Cohen, B. Razani, W. Schubert, T.M. Williams, X.B. Wang, P. Iyengar, D.L. Brasaemle, P.E. Scherer, M.P. Lisanti, Role of caveolin-1 in the modulation of lipolysis and lipid droplet formation, Diabetes 53 (2004) 1261–1270.
[257] S.M. Storey, A.L. McIntosh, S. Senthivinayagam, K.C. Moon, B.P. Atshaves, The phospholipid monolayer associated with perilipin-enriched lipid droplets is a highly organized rigid membrane structure, Am. J. Physiol. Endocrinol. Metab. 301 (2011) E991–E1003.
[258] Z. Zhou, S. Yon Toh, Z. Chen, K. Guo, C.P. Ng, S. Ponniah, S.C. Lin, W. Hong, P. Li, Cidea-deficient mice have lean phenotype and are resistant to obesity, Nat. Genet. 35 (2003) 49–56.
[259] J.Z. Li, J. Ye, B. Xue, J. Qi, J. Zhang, Z. Zhou, Q. Li, Z. Wen, P. Li, Cideb regulates diet-induced obesity, liver steatosis, and insulin sensitivity by controlling lipogenesis and fatty acid oxidation, Diabetes 56 (2007) 2523–2532.
[260] S.Y. Toh, J. Gong, G. Du, J.Z. Li, S. Yang, J. Ye, H. Yao, Y. Zhang, B. Xue, Q. Li, H. Yang, Z. Wen, P. Li, Up-regulation of mitochondrial activity and acquirement of brown adipose tissue-like property in the white adipose tissue of fsp27 deficient mice, PLoS One 3 (2008) e2890.
[261] N. Nishino, Y. Tamori, S. Tateya, T. Kawaguchi, T. Shibakusa, W. Mizunoya, K. Inoue, R. Kitazawa, S. Kitazawa, Y. Matsuki, R. Hiramatsu, S. Masubuchi, A. Omachi, K. Kimura, M. Saito, T. Amo, S. Ohta, T. Yamaguchi, T. Osumi, J. Cheng, T. Fujimoto, H. Nakao, K. Nakao, A. Aiba, H. Okamura, T. Fushiki, M. Kasuga, FSP27 contributes to efficient energy storage in murine white adipocytes by promoting the formation of unilocular lipid droplets, J. Clin. Invest. 118 (2008) 2808–2821.
[262] S. Yu, K. Matsusue, P. Kashireddy, W.Q. Cao, V. Yeldandi, A.V. Yeldandi, M.S. Rao, F.J. Gonzalez, J.K. Reddy, Adipocyte-specific gene expression and

adipogenic steatosis in the mouse liver due to peroxisome proliferator-activated receptor gamma1 (PPARgamma1) overexpression, J. Biol. Chem. 278 (2003) 498–505.
[263] K. Matsusue, T. Kusakabe, T. Noguchi, S. Takiguchi, T. Suzuki, S. Yamano, F.J. Gonzalez, Hepatic steatosis in leptin-deficient mice is promoted by the PPARgamma target gene Fsp27, Cell Metab. 7 (2008) 302–311.
[264] N. Viswakarma, S. Yu, S. Naik, P. Kashireddy, K. Matsumoto, J. Sarkar, S. Surapureddi, Y. Jia, M.S. Rao, J.K. Reddy, Transcriptional regulation of Cidea, mitochondrial cell death-inducing DNA fragmentation factor alpha-like effector A, in mouse liver by peroxisome proliferator-activated receptor alpha and gamma, J. Biol. Chem. 282 (2007) 18613–18624.
[265] Y.J. Kim, S.Y. Cho, C.H. Yun, Y.S. Moon, T.R. Lee, S.H. Kim, Transcriptional activation of Cidec by PPARgamma2 in adipocyte, Biochem. Biophys. Res. Commun. 377 (2008) 297–302.
[266] J.B. Hansen, K. Kristiansen, Regulatory circuits controlling white versus brown adipocyte differentiation, Biochem. J. 398 (2006) 153–168.
[267] V. Puri, S. Ranjit, S. Konda, S.M. Nicoloro, J. Straubhaar, A. Chawla, M. Chouinard, C. Lin, A. Burkart, S. Corvera, R.A. Perugini, M.P. Czech, Cidea is associated with lipid droplets and insulin sensitivity in humans, Proc. Natl. Acad. Sci. U.S.A. 105 (2008) 7833–7838.
[268] B. Magnusson, A. Gummesson, C.A. Glad, J.H. Goedecke, M. Jernas, T.C. Lystig, B. Carlsson, B. Fagerberg, L.M. Carlsson, P.A. Svensson, Cell death-inducing DFF45-like effector C is reduced by caloric restriction and regulates adipocyte lipid metabolism, Metab. Clin. Exp. 57 (2008) 1307–1313.
[269] J.E. Kim, S.E. Song, Y.W. Kim, J.Y. Kim, S.C. Park, Y.K. Park, S.H. Baek, I.K. Lee, S.Y. Park, Adiponectin inhibits palmitate-induced apoptosis through suppression of reactive oxygen species in endothelial cells: involvement of cAMP/protein kinase A and AMP-activated protein kinase, J. Endocrinol. 207 (2010) 35–44.
[270] J.M. Cacicedo, N. Yagihashi, J.F. Keaney Jr., N.B. Ruderman, Y. Ido, AMPK inhibits fatty acid-induced increases in NF-kappaB transactivation in cultured human umbilical vein endothelial cells, Biochem. Biophys. Res. Commun. 324 (2004) 1204–1209.
[271] N.B. Ruderman, X.J. Xu, L. Nelson, J.M. Cacicedo, A.K. Saha, F. Lan, Y. Ido, AMPK and SIRT1: a long-standing partnership? Am. J. Physiol. Endocrinol. Metab. 298 (2010) E751–E760.
[272] X. Yu, S. McCorkle, M. Wang, Y. Lee, J. Li, A.K. Saha, R.H. Unger, N.B. Ruderman, Leptinomimetic effects of the AMP kinase activator AICAR in leptin-resistant rats: prevention of diabetes and ectopic lipid deposition, Diabetologia 47 (2004) 2012–2021.
[273] A.M. Choi, K. Nakahira, Dampening insulin signaling by an NLRP3 'meta-flammasome', Nat. Immunol. 12 (2011) 379–380.
[274] H. Wen, D. Gris, Y. Lei, S. Jha, L. Zhang, M.T. Huang, W.J. Brickey, J.P. Ting, Fatty acid-induced NLRP3-ASC inflammasome activation interferes with insulin signaling, Nat. Immunol. 12 (2011) 408–415.
[275] D. Sag, D. Carling, R.D. Stout, J. Suttles, Adenosine 5′-monophosphate-activated protein kinase promotes macrophage polarization to an anti-inflammatory functional phenotype, J. Immunol. 181 (2008) 8633–8641.
[276] V.W. Dolinsky, A.Y. Chan, I. Robillard Frayne, P.E. Light, C. Des Rosiers, J.R. Dyck, Resveratrol prevents the prohypertrophic effects of oxidative stress on LKB1, Circulation 119 (2009) 1643–1652.
[277] A. Salminen, J.M. Hyttinen, K. Kaarniranta, AMP-activated protein kinase inhibits NF-kappaB signaling and inflammation: impact on healthspan and lifespan, J. Mol. Med. 89 (2011) 667–676.

[278] R. Nogueiras, K.M. Habegger, N. Chaudhary, B. Finan, A.S. Banks, M.O. Dietrich, T.L. Horvath, D.A. Sinclair, P.T. Pfluger, M.H. Tschop, Sirtuin 1 and sirtuin 3: physiological modulators of metabolism, Physiol. Rev. 92 (2012) 1479–1514.
[279] A. Chalkiadaki, L. Guarente, High-fat diet triggers inflammation-induced cleavage of SIRT1 in adipose tissue to promote metabolic dysfunction, Cell Metab. 16 (2012) 180–188.
[280] Z. Yang, B.B. Kahn, H. Shi, B.Z. Xue, Macrophage alpha1 AMP-activated protein kinase (alpha1AMPK) antagonizes fatty acid-induced inflammation through SIRT1, J. Biol. Chem. 285 (2010) 19051–19059.
[281] F. Liang, S. Kume, D. Koya, SIRT1 and insulin resistance, Nat. Rev. Endocrinol. 5 (2009) 367–373.
[282] Y. Peng, D.A. Rideout, S.S. Rakita, W.R. Gower Jr., M. You, M.M. Murr, Does LKB1 mediate activation of hepatic AMP-protein kinase (AMPK) and sirtuin1 (SIRT1) after Roux-en-Y gastric bypass in obese rats? J. Gastrointest. Surg. 14 (2010) 221–228.
[283] M. Troseid, I. Seljeflot, H. Arnesen, The role of interleukin-18 in the metabolic syndrome, Cardiovasc. Diabetol. 9 (2010) 11.
[284] S. de Ferranti, D. Mozaffarian, The perfect storm: obesity, adipocyte dysfunction, and metabolic consequences, Clin. Chem. 54 (2008) 945–955.
[285] P. Calabro, D.W. Chang, J.T. Willerson, E.T. Yeh, Release of C-reactive protein in response to inflammatory cytokines by human adipocytes: linking obesity to vascular inflammation, J. Am. Coll. Cardiol. 46 (2005) 1112–1113.
[286] M.P. Stein, J.C. Edberg, R.P. Kimberly, E.K. Mangan, D. Bharadwaj, C. Mold, T.W.Du. Clos, C-reactive protein binding to FcgammaRIIa on human monocytes and neutrophils is allele-specific, J. Clin. Invest. 105 (2000) 369–376.
[287] A.R. Sinaiko, J. Steinberger, A. Moran, R.J. Prineas, B. Vessby, S. Basu, R. Tracy, D.R. Jacobs Jr., Relation of body mass index and insulin resistance to cardiovascular risk factors, inflammatory factors, and oxidative stress during adolescence, Circulation 111 (2005) 1985–1991.
[288] M.D. DeBoer, M.J. Gurka, A.E. Sumner, Diagnosis of the metabolic syndrome is associated with disproportionately high levels of high-sensitivity C-reactive protein in non-Hispanic black adolescents: an analysis of NHANES 1999–2008, Diabetes Care 34 (2011) 734–740.
[289] A.C. Oliveira, A.M. Oliveira, L.F. Adan, N.F. Oliveira, A.M. Silva, A.M. Ladeia, C-reactive protein and metabolic syndrome in youth: a strong relationship? Obesity (Silver Spring) 16 (2008) 1094–1098.
[290] R. Retnakaran, A.J. Hanley, P.W. Connelly, S.B. Harris, B. Zinman, Elevated C-reactive protein in Native Canadian children: an ominous early complication of childhood obesity, Diabetes Obes. Metab. 8 (2006) 483–491.
[291] M. Siervo, D. Ruggiero, R. Sorice, T. Nutile, M. Aversano, M. Iafusco, F. Vetrano, J.C. Wells, B.C. Stephan, M. Ciullo, Body mass index is directly associated with biomarkers of angiogenesis and inflammation in children and adolescents, Nutrition 28 (2012) 262–266.
[292] E.S. Ford, U.A. Ajani, A.H. Mokdad, The metabolic syndrome and concentrations of C-reactive protein among U.S. youth, Diabetes Care 28 (2005) 878–881.
[293] N. Mattsson, T. Ronnemaa, M. Juonala, J.S. Viikari, O.T. Raitakari, Childhood predictors of the metabolic syndrome in adulthood. The Cardiovascular Risk in Young Finns Study, Ann. Med. 40 (2008) 542–552.
[294] M.J. Jarvisalo, A. Harmoinen, M. Hakanen, U. Paakkunainen, J. Viikari, J. Hartiala, T. Lehtimaki, O. Simell, O.T. Raitakari, Elevated serum C-reactive protein levels and early arterial changes in healthy children, Arterioscler. Thromb. Vasc. Biol. 22 (2002) 1323–1328.

[295] A.A. Meyer, G. Kundt, M. Steiner, P. Schuff-Werner, W. Kienast, Impaired flow-mediated vasodilation, carotid artery intima-media thickening, and elevated endothelial plasma markers in obese children: the impact of cardiovascular risk factors, Pediatrics 117 (2006) 1560–1567.
[296] S.K. Fried, D.A. Bunkin, A.S. Greenberg, Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid, J. Clin. Endocrinol. Metab. 83 (1998) 847–850.
[297] A.W. Taylor, N.O. Ku, R.F. Mortensen, Regulation of cytokine-induced human C-reactive protein production by transforming growth factor-beta, J. Immunol. 145 (1990) 2507–2513.
[298] C. Platat, A. Wagner, T. Klumpp, B. Schweitzer, C. Simon, Relationships of physical activity with metabolic syndrome features and low-grade inflammation in adolescents, Diabetologia 49 (2006) 2078–2085.
[299] S.V. Galcheva, V.M. Iotova, Y.T. Yotov, S. Bernasconi, M.E. Street, Circulating proinflammatory peptides related to abdominal adiposity and cardiometabolic risk factors in healthy prepubertal children, Eur. J. Endocrinol. 164 (2011) 553–558, European Federation of Endocrine Societies.
[300] A. Alikasifoglu, N. Gonc, Z.A. Ozon, Y. Sen, N. Kandemir, The relationship between serum adiponectin, tumor necrosis factor-alpha, leptin levels and insulin sensitivity in childhood and adolescent obesity: adiponectin is a marker of metabolic syndrome, J. Clin. Res. Pediatr. Endocrinol. 1 (2009) 233–239.
[301] S. Maumus, B. Marie, G. Siest, S. Visvikis-Siest, A prospective study on the prevalence of metabolic syndrome among healthy french families: two cardiovascular risk factors (HDL cholesterol and tumor necrosis factor-alpha) are revealed in the offspring of parents with metabolic syndrome, Diabetes Care 28 (2005) 675–682.

CHAPTER FIVE

CSF in Alzheimer's Disease

Henrik Zetterberg[*,†,1], **Ronald Lautner**[*], **Tobias Skillbäck**[*], **Christoffer Rosén**[*], **Pashtun Shahim**[*], **Niklas Mattsson**[*,‡,§], **Kaj Blennow**[*]

[*]Clinical Neurochemistry Laboratory, Institute of Neuroscience and Physiology, The Sahlgrenska Academy at the University of Gothenburg, Mölndal, Sweden
[†]UCL Institute of Neurology, Queen Square, London, United Kingdom
[‡]Department of Veterans Affairs Medical Center, Center for Imaging of Neurodegenerative Diseases, San Francisco, California, USA
[§]Department of Radiology and Biomedical Imaging, University of California, San Francisco, California, USA
[1]Corresponding author: e-mail address: henrik.zetterberg@gu.se

Contents

Abstract

Alzheimer's disease (AD) is a progressive brain amyloidosis that injures brain regions involved in memory consolidation and other cognitive functions. Neuropathologically, the disease is characterized by accumulation of a 42-amino acid protein called

Advances in Clinical Chemistry, Volume 65
ISSN 0065-2423
http://dx.doi.org/10.1016/B978-0-12-800141-7.00005-X

amyloid β, and N-terminally truncated fragments thereof, in extracellular senile plaques together with intraneuronal inclusions of hyperphosphorylated tau protein in neurofibrillary tangles, and neuronal and axonal degeneration and loss. Clinical chemistry tests for these pathologies have been developed for use on cerebrospinal fluid samples. Here, we review what these markers have taught us on the disease process in AD and how they can be implemented in routine clinical chemistry. We also provide an update on new marker development and ongoing analytical standardization effort.

ABBREVIATIONS

AD Alzheimer's disease
APP amyloid precursor protein
Aβ42 amyloid β 42
BACE1 beta-site APP-cleaving enzyme 1
CCL2 C—C chemokine ligand 2
CNS central nervous system
CSF cerebrospinal fluid
CV coefficient of variation
ELISA enzyme-linked immunosorbent assay
fAD familial AD
GCBS Global Consortium for Biomarker Standardization
HIV human immunodeficiency virus
IFCC International Federation of Clinical Chemistry and Laboratory Medicine
IL-1β interleukin 1β
MCI mild cognitive impairment
NFL neurofilament light protein
P-tau phospho-tau
QC quality control
sAPPα soluble APP alpha
sAPPβ soluble APP beta
TGF-β transforming growth factor-β
TNF-α tumor necrosis factor α
T-tau total tau

1. INTRODUCTION

In 1906, Alzheimer described the clinical characteristics of a female patient in her 50s with a progressive memory disorder that eventually ended her life [1]. The neuropathology was striking with three main features: (i) gross atrophy, which today is known to be due to neuronal and synaptic/axonal degeneration and loss, (ii) extracellular argyrophilic accumulations called senile plaques, and (iii) intraneuronal inclusions called

neurofibrillary tangles [2]. In 1911, Kraepelin named this condition Alzheimer's disease (AD) in his influential textbook on psychiatry. For many decades, AD was considered a rare brain disorder found in patients who developed dementia in middle age. However, during the 1960s, it was noted that many elderly who died demented actually displayed neuropathological changes similar to those of AD [3], and the term senile dementia of Alzheimer type was coined. Thereafter, the distinction between early-onset AD and senile dementia of the Alzheimer type faded out, and the term AD was introduced as the common term for both entities [4].

In 1966, Roth and colleagues found a positive correlation between plaque counts and how demented the patient had been prior to death [5], which stimulated research on the molecular composition of the plaque. In 1984, Glenner and Wong managed to purify and partially sequence a protein derived from twisted β-pleated sheet fibrils in cerebrovascular amyloidosis of AD brains [6]. They called the protein amyloid fibril protein β. The year after, Masters and colleagues established the presence of an around 40-amino acid protein, with a sequence similar to what Glenner and Wong had reported, in plaques from AD and Down's syndrome brains [7]. Masters and Beyreuther called it the A4 protein because of its 4 kDa molecular weight. This term has now virtually disappeared in favor of amyloid β (Aβ), and we know that the predominating Aβ forms in senile plaques are Aβ4–42 (Aβ starting at amino acid 4 in the Aβ domain and ending at amino acid 42 after a stretch of hydrophobic amino acids making the protein self-adhesive), Aβ1–42, and Aβ1–40, with some pyroglutamate-modified Aβ3–42 [8].

In 1986, 1 year after the sequencing of Aβ in plaques, data were published showing that an abnormally hyperphosphorylated form of tau protein is the main component of neurofibrillary tangles [9,10]. Tau is a microtubule-binding axonal protein that promotes microtubule assembly and stability. Abnormal phosphorylation and truncation of tau may lead to disassembly of microtubules and impaired axonal transport with compromised neuronal function and tau aggregation into paired helical filaments and neurofibrillary tangles [11].

2. AD—GENETICS AND PATHOPHYSIOLOGY

The identification of Aβ as the main component of plaques initiated a search for the gene from which it is coded. In 1987, the gene of the precursor

protein (amyloid precursor protein, APP) was cloned, sequenced, and localized to chromosome 21 [12,13]. A gene-dose effect of the triplication of the *APP* gene in Down's syndrome (trisomy 21) is thought to explain plaque pathology in early adulthood in this condition, although some studies show a similar frequency of pathology also in mental retardation of other causes, without an extra *APP* gene [14].

APP is a type I transmembrane protein with one membrane-spanning domain and is expressed not only in the brain but also in other compartments of the body. The secreted form of APP was soon found to be identical to an already known plasma antichymotrypsin protease involved in coagulation, nexin-2 [15]. The exact function of APP in the brain remains elusive, but the protein appears to be involved in several biological processes, such as brain development, synaptic plasticity, and neuroprotection [16]. Mutations in the *APP* gene were found in familial AD (fAD) cases [17–19], all of which influenced Aβ production in an amyloidogenic manner. The enzymes responsible for amyloidogenic APP processing (β- and γ-secretase) were identified, and causative fAD mutations were found in the presenilin genes that encode the proteins that build up the active site of γ-secretase [20]. There are now convincing data suggesting that most fAD-causing presenilin mutations (there are at least 170) lead to γ-secretase dysfunction so that relatively more Aβ42 is produced when dysfunctional γ-secretase does not manage to process amyloidogenic Aβ42 to less aggregation-prone Aβ37/38/39 variants [21], thus promoting brain amyloidosis. That mutations in both the substrate (APP) and one of the key enzymes (γ-secretase) can cause AD by promoting brain amyloidosis and that different preparations of Aβ kill neurons and/or inhibit long-term potentiation (an electrophysiological correlate to memory consolidation) when applied to hippocampal slices or neuronal cell cultures, or when injected into the central nervous system (CNS) of rodents [22] have fueled the notion that Aβ accumulation actually drives the disease process in AD, and that tangle formation (tau pathology) and neurodegeneration are downstream events. This view has been summarized in the amyloid cascade hypothesis on AD [23], which is still highly relevant with minor modifications [24]. One common criticism against the hypothesis is that it is based primarily on mechanisms operating in fAD, and it is not clear how relevant these are for sporadic AD, which (by definition) does not have autosomal dominant hereditability and includes the vast majority of patients diagnosed with AD. Specifically, it has been proposed that the cognitive decline may have more heterogeneous causes in late onset AD compared to early-onset AD [25]. However, the

importance of Aβ pathology for the development of sporadic AD was recently supported by the identification of a rare protective *APP* mutation on Iceland that changes amino acid 673 in APP from alanine to threonine (position 2 in the Aβ domain) and results in decreased β-secretase-mediated Aβ production [26].

The genetic component is important not only in fAD but also in sporadic AD. Twin studies suggest that 40–70% of the risk of sporadic AD is explained by genetic factors [27,28]. The most important susceptibility gene for sporadic AD is the *APOE* ε4 gene variant, which accounts for around 50% of the risk [29], but many more low-risk loci exist [30]. Genome-wide association studies have identified susceptibility genes linked to at least three molecular pathways that may be involved in AD pathogenesis: (i) endosomal vesicle recycling, (ii) cholesterol metabolism, and (iii) the innate immune system [31]. Further, exome sequencing has identified a rare, heterozygous loss-of-function mutation in a microglia-regulating gene (*TREM2*) that renders microglia overactive to brain amyloidosis and increases the risk of clinical AD at least fourfold [32,33]. It is not yet possible to definitively relate these pathways to each other or to APP but some suggestions emerge from the literature and they may all be targets for therapy.

As described above, and as supported by genetic and neuropathological data, the definitions of AD and old age dementia have changed dramatically during the last century, with AD going from being a rare cause of early-onset dementia to a broad diagnosis including most patients with age-related dementia. However, despite this development, there is also a debate regarding the relationship between AD and normal aging, and regarding the relative roles of the different pathological hallmarks of AD to clinical presentation of the disease. At the core of this debate are some facts that are difficult to reconcile, including that (i) brain atrophy is widespread in normal elderly subjects, also in brain regions primarily associated with AD, including the hippocampus [34], (ii) autopsy studies indicate that Aβ plaque pathology appears first in neocortical association areas, and only later in the hippocampus [35], (iii) tau pathology may appear decades before plaque pathology in the population [36], (iv) the location and spread of tau pathology are more closely related to the cognitive loss in AD than the location and spread of Aβ pathology [37], and (v) mutations in the *MAPT* gene, which encodes the tau protein, most often cause frontotemporal lobe degeneration and not AD (but there are exceptions to this [38,39]), while mutations in *APP* and presenilin genes are generally described to cause AD rather than frontotemporal lobe degeneration.

The controversy regarding the roles of plaques and tangles can be traced back even to the work of Alzheimer himself, who first presented a description of a demented patient with combined plaque and tangle pathology, as described above [1], but who later published a lengthy description of a patient (a 56-year-old male) with "plaque-only" dementia (translated to English in Ref. [40]). In this second paper, Alzheimer concluded that the plaques were similar in young and elderly dementia patients "It cannot be doubted that the plaques in these specific cases do in all relevant aspects correspond to those which we find in Dementia senilis.", and he observed a poor spatial correlation between plaques and other degenerative changes, leading him to suggest that "the plaques are not the cause of senile dementia but only an accompanying feature of senile involution of the central nervous system." Later studies suggest that "plaque-only" dementia patients often have combinations of Aβ plaques and other pathologies [41]. The clarification how the pathologies seen in different neurodegenerative diseases and in normal aging are related to each other will likely influence our future definitions of AD. Hypothetically, one possibility is that different pathologies may arise partly independent of each other, with different pathologies appearing first in different people. These pathologies may include not only protein aggregates such as Aβ plaques, tau tangles, and Lewy bodies composed of α-synuclein but also a continuous normal loss of neurons or synapses unrelated to the presence of inclusion bodies. The different brain changes may converge and interact to cause both local and widespread functional and structural changes in the brain, ultimately resulting in cognitive dysfunction sometimes recognized as AD. Recently, the identification of tau deposits in select subcortical nuclei in young persons, decades before amyloid accumulation starts [42], has vitalized the idea that tau and Aβ pathologies may start independent from each other.

3. CSF IN AD

Cerebrospinal fluid (CSF) is located in the cerebral ventricles and also surrounds the brain. It communicates freely with the brain interstitial fluid and may thereby serve as a biochemical window into the brain. CSF investigations in AD were pioneered by Gottfries and colleagues in the late 1960s and early 1970s, who reported reduced CSF monoamine metabolite concentrations, suggesting a breakdown of these neurotransmitter systems in the deteriorating brain [43,44], and elevated CSF lactate concentration as a sign of tissue hypoxia [45]. During the 1990s, tests for proteins thought

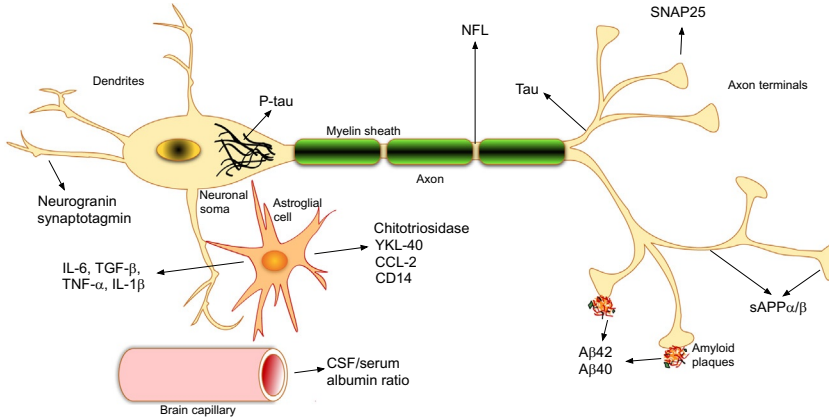

Figure 5.1 The figure depicts a tangle-bearing neuronal soma with dendrites, axon, and axon terminals. An astroglial cell, a blood vessel, and senile plaques are also represented. Key biomarkers for different pathologies and cell compartments, discussed in detail in the text, are indicated.

to reflect the core neuropathology of AD were developed. Established and candidate biomarkers are discussed below and a summary is given in Fig. 5.1.

3.1. CSF Aβ42

Initially, just after the identification of aggregation-prone Aβ proteins ending at amino acids 40 and 42 in senile plaques [6,7,46], the protein was thought to be an abnormal side-product of APP metabolism invariably associated with AD. The natural secretion of Aβ from untransfected primary cells therefore came as a surprise [47]. Since then, it has been established that APP can enter at least three proteolytic clearance pathways: (i) amyloidogenic processing that primarily leads to production of Aβ42 and Aβ40 (but also some shorter, less aggregation-prone fragments) by β- and γ-secretase cleavages, (ii) nonamyloidogenic processing that leads to production of sAPPα and possibly also a C-terminal fragment called p3 (this fragment should consist of Aβ17–40/42, but has been difficult to verify using modern techniques; it is possible that it is quickly degraded into shorter fragments), and (iii) another nonamyloidogenic processing pathway involving concerted cleavages of APP by β- and α-secretase resulting in production of Aβ1–14/15/16 fragments at the expense of longer Aβ fragments [48].

The first assay for CSF Aβ42 was published in 1995 [49]. Using this enzyme-linked immunosorbent assay (ELISA), AD patients had reduced levels of CSF Aβ42, which has been verified in hundreds of papers [50].

The reduction reflects Aβ42 retention in senile plaques in the brain, as evidenced by both autopsy and *in vivo* imaging studies [51–54]. In 1999, the first paper showing a reduction in CSF Aβ42 in mild cognitive impairment (MCI) patients who later progressed to AD dementia was published [55]. Since then, numerous studies have verified that low CSF Aβ42 levels are highly predictive of future AD, both in MCI [56–59] and cognitively normal cohorts [60–62]. In fact, CSF Aβ42 is the earliest nongenetic biomarker we know of in AD. Low CSF Aβ42 concentrations in the absence of senile plaques have been reported in neuroinflammatory conditions, for example, bacterial meningitis [63], multiple sclerosis [64], human immunodeficiency virus (HIV)-associated dementia [65], and Lyme neuroborreliosis [66], and are often accompanied by biomarker evidence of a general reduction in APP metabolites, for example, secreted forms of APP, which is not typical of AD [50].

Besides Aβ42, several other Aβ isoforms are present in CSF. The most abundant variant in CSF is Aβ40, which is relatively unchanged in AD. The ratio of CSF Aβ42 to Aβ40 has been suggested to have stronger diagnostic accuracy for AD compared to CSF Aβ42 alone [67]. There are also several other C- or N-terminally truncated Aβ isoforms in CSF, which may be altered in AD [68], including in early clinical stages [69].

3.2. CSF T-tau

The first CSF total tau (T-tau) assay was published in 1993 [70]. This assay was a sandwich ELISA in which a monoclonal antibody against the mid-domain of tau was combined with a polyclonal anti-tau antiserum. Two years later, the first assay based on three mid-region monoclonal antibodies, which recognizes all tau isoforms irrespective of phosphorylation state, was published [71], today known as the "Innogenetics assay." AD patients displayed clearly elevated T-tau levels [70,71], a finding that has been replicated in hundreds of papers, using several different assays, in many different clinical contexts [50]. It has been shown that CSF T-tau levels correlate with imaging measures of hippocampal atrophy [72] and gray matter degeneration [73], which would be logical given the high expression of tau in thin unmyelinated axons of the cortex [74]. In response to acute brain injury, CSF T-tau levels are dynamic; they increase during the first few days following the injury and stay elevated for a few weeks until they normalize [75,76]. This has led to the view that elevated CSF T-tau levels reflect ongoing axonal degeneration, which in turn may indicate disease intensity. Indeed, CSF

T-tau predicts the malignancy of the clinical course in AD; the higher levels, the more rapid clinical disease progression [77]. The most extreme neurodegenerative condition we are aware of, Creutzfeldt–Jakob disease, goes with very pronounced CSF T-tau elevations that are often orders of magnitude higher than what is typically seen in AD [78].

Two recent discoveries have made the tau biomarker field a bit more complicated: (i) the finding of tau secretion from cultured cells [79] and mouse neurons [80] in a manner that may be stimulated by Aβ in the absence of frank neuronal death [80], and (ii) the finding that most of tau in CSF is fragmented [81]. Tau secretion should not have come as a surprise, as it has been well known for decades that healthy individuals have tau in their CSF, although at lower concentrations than the typical AD or other brain injury patient [82], but still, it makes it more challenging to interpret CSF T-tau levels. Perhaps, the AD process somehow induces increased tau expression and release and that this, not neuronal death, is the primary reason for tau elevation in AD? This view would fit with tau changes in other forms of brain injury. Following acute experimental brain injury in animals, an intraneuronal increase in tau expression has been demonstrated [83]. The other finding, that most CSF tau is fragmented goes well with the established presence of endogenous tau fragments in tangles [84], explains why it has been important to have capture and detection antibodies located closely to each other in T-tau assays, and why combinations of distally located N- and C-terminal antibodies do not work [81]. Tau fragments also present a challenge when we want to create reference methods and materials for this marker. How should we define the standard protein to be measured in such materials?

3.3. CSF P-tau

The first CSF assay for phosphorylated tau (P-tau), the form of tau that is thought to represent neurofibrillary tangles, was published in 1995 [71]. Since then, P-tau assays for different forms of phosphorylated tau proteins have been examined [85]. They correlate well and associate with AD in a similar manner [85]. CSF P-tau levels correlate with neurofibrillary tangle pathology [54,86]. The major outstanding research question regarding P-tau assays is why other tauopathies, like frontotemporal dementias and progressive supranuclear palsy, do not show P-tau elevation, at least not as systematically as AD does. It is possible that these disorders show disease-specific tau phosphorylation or that tau is processed or truncated

in a way that is not recognized by available assays, which is an area in need of further research. In the meantime, we should make use of the AD specificity for P-tau elevations. There are at present only three conditions in addition to AD in which elevated CSF P-tau has been reported: (i) in term and preterm newborns, possibly reflecting physiological tau phosphorylation in brain development [87], (ii) in herpes encephalitis [88], and (iii) in superficial CNS siderosis [89,90]. Clearly, these conditions are no important differential diagnoses to AD, but they may shed light on mechanisms behind CSF P-tau increase (as may the data on tau phosphorylation in hibernating squirrels [91] and hamsters [92]).

3.4. Diagnostic performance of combined CSF T-tau, P-tau, and Aβ42 tests

Multiple studies have investigated the diagnostic accuracy of combined CSF tests for T-tau, P-tau, and Aβ42 [82]. These studies collectively point to sensitivities and specificities of 85–95% in cross-sectional AD-control studies, as well as in longitudinal studies of patients fulfilling MCI criteria [82]. Higher diagnostic performance is typically seen in monocenter studies [56,93], whereas large multicenter studies tend to report slightly lower sensitivities and specificities [58,94]. The association of elevated T-tau and P-tau and reduced Aβ42 with AD neuropathology has been validated in autopsy [57] and brain biopsy [54] studies. Interestingly, the alterations in CSF Aβ42, T-tau, and P-tau are similar between normal AD, where the cognitive dysfunction is dominated by episodic memory loss, and a rarer variant of AD (posterior cortical atrophy), where visuospatial dysfunction dominates [95,96], but this topic has been less thoroughly explored in other clinical variants of AD, including AD presenting with logopenic primary progressive aphasia [97] or corticobasal syndrome [98].

3.5. Longitudinal changes in CSF AD biomarkers and usage in clinical trials

Recent data show that it is possible to identify longitudinal changes in CSF Aβ42, T-tau, and P-tau in cognitively healthy controls followed with multiple lumbar punctures over several years [99–101], but most studies (with exceptions [99]) show that CSF AD biomarkers are essentially stable in symptomatic AD [69,102,103]. This biomarker stability (at least during short-term follow-up) may be useful in clinical trials to help identify effects of interventions, both on the intended biological target, such as altered Aβ metabolism in response to an anti-Aβ treatment. One of the truly

longitudinal studies of cognitively normal individuals with repeated CSF samples suggests that Aβ42 and tau changes occur in parallel and predict upcoming cognitive symptoms better than absolute baseline levels [101]. CSF measurements may track trajectories of specific Aβ and APP metabolites [104–107], and downstream effects on secondary phenomena, such as reduced axonal degeneration in response to a disease-modifying drug as measured by CSF tau levels [108,109].

4. CANDIDATE AD BIOMARKERS AND MARKERS OF OTHER PATHOLOGIES

As explained above, AD is sometimes described as a heterogeneous condition with a phenotype that can be mimicked by various aging processes in the brain. Clinically, AD in an 85-year-old person with type II diabetes, hypertension, and sleep apnea is most likely not the same condition as AD in an otherwise healthy 60-year-old with a positive family history. There are also several other conditions that may contribute to AD-like cerebral dysfunction [110]. A recent clinicopathologic study reported the following most prevalent AD mimics: dementia with Lewy bodies, cerebrovascular disease, frontotemporal lobar degeneration, and hippocampal sclerosis [111]. Finally, atrophy in the presence of brain plaque and tangle pathology may sometimes strike brain regions in an atypical manner, which may cause symptoms that on clinical or neuroimaging grounds are grouped as, for example, posterior cortical atrophy and logopenic or progressive nonfluent aphasia, which in some cases are believed to represent variant clinical presentations of AD [110]. A vision in the biomarker research field is to develop biomarkers that can be used as adjuncts to neuroimaging and neuropsychology measures to increase the understanding of the molecular basis of the phenotype of the patient.

4.1. CSF BACE1

As described above, in the amyloidogenic pathway, Aβ is produced through proteolytic processing of APP by β-and γ-secretases. The major β-secretase in the brain is the β-site APP-cleaving enzyme 1 (BACE1) [112]. Increased BACE1 activity has been measured in postmortem samples from patients with AD [113]. The activity and concentration of BACE1 can also be measured in CSF, but the results have been conflicting. Holsinger *et al.* found increased activity of CSF BACE1 in AD as well as in other dementias [114,115]. The activity of CSF BACE1 was also found to be elevated in

patients with MCI who progressed to AD compared to subjects with MCI who remained stable or developed other forms of dementia [116]. Many studies, however, have failed to show any significant difference in BACE1 activity between MCI and AD patients compared to controls [117,118]. One study suggests that CSF BACE1 activity may drop in advanced disease stages [119]. Altogether, the discrepant results of these studies suggest that the diagnostic value of BACE1 in AD is limited. However, the marker may still be valuable in clinical trials of BACE1 inhibitors.

4.2. CSF sAPPα/sAPPβ

Theoretically, secreted forms of APP should be excellent fluid markers of amyloidogenic (sAPPβ) and nonamyloidogenic (sAPPα) APP processing. The proteins are readily measureable in CSF, but several studies have failed to show any differences between AD patients and controls [93,116,120]. In the context of MCI, one study reported elevated CSF sAPPβ in patients with MCI versus controls [120], and another study showed increased CSF sAPPβ in MCI patients who progressed to AD versus patients who remained stable [121]. Studies that grouped patients on the basis of CSF tau and Aβ markers into neurochemical AD and non-AD groups found elevated CSF sAPPα and sAPPβ in AD, but did not take into account that the differences might have been driven by lower levels in the non-AD disease groups (there were no cognitively normal controls in these studies.) [122–124]. Similar to BACE1, the results of studies exploring CSF sAPPα and sAPPβ as potential biomarkers for AD have been inconsistent. However, these biomarkers may be valuable in clinical trials to monitor the effect of novel therapies targeting APP metabolism.

4.3. Aβ oligomers

The correlation between cerebral load of amyloid plaques and disease severity is poor [125]. To reconcile this observation with the amyloid cascade hypothesis, it has been suggested that most of the presumed Aβ toxicity is exerted by soluble portions of Aβ, while the plaques *per se* are relatively inert. Walsh *et al.* showed that soluble oligomers of Aβ inhibit hippocampal long-term potentiation in rats [126], and others have found that they can lead to abnormal phosphorylation of tau as well as neuritic dystrophy [127–129]. Comparing AD with controls, studies have shown that the former have higher levels of oligomers in their brains [130,131]. Several studies have tried to replicate these findings in CSF, but results have been inconsistent.

A problem is that oligomers occur only in minute amounts in CSF, making them difficult to reliably quantitate. Some studies have found elevated CSF Aβ oligomer in AD [132–136] or in cognitively normal older adults with an AD-like biomarker profile [137], while others have not found this relationship [130,138]. The term "oligomer" includes species from dimers to much larger protofibrils, and this may be an explanation to the diverging results, since most studies have not used methods that enable a precise characterization of the measurand.

4.4. Blood–brain barrier biomarkers

The best-established biomarker so far for the integrity of the blood–brain barrier is the ratio of albumin in CSF to serum (the CSF/serum albumin ratio). Typically, the CSF/serum albumin ratio is normal in patients with pure AD [139], whereas patients with cerebral small vessel disease generally present with increased CSF/serum albumin ratio [140]. The same finding is often present in Lyme disease (neuroborreliosis), where one also may find increased numbers of CSF monocytes and signs of immunoglobulin production within the CNS [141]. Novel potential but so far less well-established blood–brain biomarkers in CSF include secretory Ca^{2+}-dependent phospholipase A2 activity [142] and antithrombin III [143]. More research is warranted in this field, especially to develop assays to differentiate between specific forms of blood–brain barrier dysfunction.

4.5. Biomarkers for subcortical axonal degeneration

An established biomarker of the subcortical axonal degeneration frequently seen in potential AD mimics, such as cerebral small vessel disease [144–146], frontotemporal lobar degeneration [147,148], and HIV-associated dementia [149], is neurofilament light protein (NFL). NFL, as well as other members of the neurofilament group of proteins, acts as an integral part of the neural cytoskeleton, providing structural support for predominantly large-caliber myelinated axons. Elevated CSF NFL indicates involvement of these axons in the disease process and helps differentiate pure AD from the conditions listed above. Combined T-tau and NFL increases are common in mixed forms of AD and cerebrovascular disease. These mixed CSF findings are very common in unselected patients undergoing evaluation because of suspected neurodegenerative disease [150]. NFL is also a useful biomarker for damage severity in several other conditions characterized by white matter lesions and

injury to subcortical brain regions such as idiopathic normal pressure hydrocephalus [151], ALS [152], various CNS infections [88], and stroke [153].

4.6. Biomarkers for inflammation, oxidative stress, and microglial activation

Inflammation, oxidative stress, and microglial activation in AD may be downstream phenomena of neurodegeneration, but recent genetic data suggest that they may well contribute to pathogenesis in susceptible individuals [32,33]. Possible triggers are the accumulation of abnormal proteins (aggregated Aβ in the case of AD) and/or mediators released from dying cells. Such triggers may lead to overshoot inflammation in some individuals, for example, carriers of a recently described loss-of-function mutation in the microglia-controlling triggering receptor expressed on myeloid cells-2 (*TREM2*) gene [32,33], possibly making them more likely to develop clinical AD in response to Aβ.

Many studies have examined potential biomarkers linked to inflammatory processes. Cytokines, such as interleukin 6, transforming growth factor (TGF-β), tumor necrosis factor α (TNF-α), and interleukin 1β (IL-1β), have been measured in CSF of AD patients, but upon meta-analysis in 2010, the only consistent finding was increased CSF levels of TGF-β in AD versus control groups [154]. Additional candidate inflammatory biomarkers that have been examined in relation to AD since then include the cytokine osteopontin, which is elevated in CSF from AD patients [155], and the TNF-α-induced proinflammatory agent lipocalin 2 which has been found at lower concentrations in CSF from MCI and AD patients compared with controls [156]. Another study found that CSF lipocalin 2, also known as neutrophil gelatinase-associated lipocalin, occurred at lower levels in AD and stable MCI patients compared with patients who had AD and vascular risk factors [157].

It should be noted that the biomarker literature is tainted by studies reporting CSF cytokine or interleukin concentrations below the analytical sensitivity of the employed assays (IL-1β is just one example) and that standard clinical chemistry tests for neuroinflammation, including CSF leukocyte count and general signs of IgG or IgM production within the CNS, are generally negative in AD and other primary neurodegenerative diseases [158]. Distinct positive results on these latter tests speak against pure AD and should motivate further investigation of the patient to exclude neuroborreliosis, multiple sclerosis, and other neuroinflammatory conditions that may contribute to the cognitive symptoms.

Isoprostanes, in particular a subclass called F2-isoprostanes, are the most examined CSF biomarkers for oxidative stress. They are prostaglandin-like compounds produced by free radical-dependent peroxidation of arachidonic acid [159]. Studies report elevated F2-isoprostane levels in AD CSF [160–164] in a manner that appears to be downstream of Aβ pathology [165]. CSF isoprostanes correlate to clinical disease progression in the MCI and dementia stages of AD, especially in *APOE* ε4-carrying patients [166], and may serve as damage response markers. Pilot studies suggest that the levels of oxidative DNA damage repair products are elevated in CSF from mixed vascular and Alzheimer's dementia patients [167], and that reduced levels of mitochondrial DNA in CSF suggest depletion of mitochondria [168], which may reflect oxidative stress, but these studies await replication.

Neuroinflammation is tightly linked to activation of the inflammatory M1 phenotype of microglia, the macrophages of the brain. Chitotriosidase is an enzyme that is secreted by activated macrophages [169] and its plasma levels are increased in patients with the lysosomal storage disorder, Gaucher disease [170]. Increased CSF chitotriosidase activity has been found in AD versus nondemented controls [171]. A glycoprotein that has great homology with chitotriosidase but lacks its enzymatic activity is YKL-40 [172]. YKL-40 is expressed in both microglia and astrocytes and elevated levels have been reported in both prodromal AD and cerebrovascular disease [173,174].

Another microglial marker, the C—C chemokine receptor 2, is expressed on monocytes and one of its ligands, C—C chemokine ligand 2 (CCL2), that can be produced by microglia is important for the recruitment of monocytes in the CNS [175]. Increased CSF CCL2 has been associated with a faster cognitive decline in MCI patients who developed AD [176]. CCL2 levels in CSF were increased in AD patients compared with healthy controls [177,178], as well as in the MCI stage of the disease [179]. However, one study failed to report any significant differences between AD patients and controls [180]. Another study found increased CSF CCL2 in AD patients versus controls, but there was an age-dependent increase that may have affected the result [181]. Moreover, one study reported elevated levels of a soluble form of CD14 in the CSF from AD (and Parkinson's disease, PD) versus healthy controls [182]. CD14 is a surface protein mainly expressed by macrophages. As a cofactor for Toll-like receptors, CD14 is essential for the recognition of pathogens by the innate immune system of the brain. Another microglial biomarker that has been detected in the CSF of AD patients is neopterin, a degradation product

deriving from the purine nucleotide guanosine triphosphate. However, no significant differences between AD and controls have been seen to date [183].

Taken together, biomarker studies support involvement of low-grade neuroinflammation, oxidative stress, and microglial activation in the AD process. Future longitudinal studies of healthy individuals will most likely help to sort out in what order these markers change in relation to plaque and tangle pathology and neurodegeneration in AD. A recent study found that several proteins in CSF, possibly associated with microglia activity, predicted longitudinal reduction of CSF Aβ42 in cognitively healthy subjects, suggesting involvement of inflammatory pathways in early AD [100].

4.7. Biomarkers for synaptic changes

Loss of synapses is highly correlated with decrease in neurocognitive function in AD patients [184]. A biomarker that reflects this pathology would therefore be desirable. Synaptic proteins such as synaptotagmin, growth-associated protein 43, synaptosomal-associated protein 25, rab3a, and neurogranin are abundant in brain tissue, but present at very low concentrations in CSF [185], thus presenting an analytical challenge. Nevertheless, the dendritic protein neurogranin has been detected in CSF and elevated levels in AD have been observed using a semi-quantitative immunoblot method [186]. More research is needed to determine the biomarker potential of synaptic proteins in the CSF. Recent breakthroughs in ultrasensitive immunochemical techniques may help in this regard [187,188].

4.8. Other protein inclusions

α-Synuclein is the major component of intraneuronal Lewy bodies, which are characteristic of PD and dementia with Lewy bodies [189]. α-Synuclein pathology is sometimes found together with Aβ plaques and neurofibrillary tangles in AD [190]. In addition, experimental studies show that Aβ42 enhances aggregation of α-synuclein [191]. CSF α-synuclein in PD and other synucleinopathies is typically reduced [192,193]. In AD and CJD, the levels are elevated and correlate to T-tau, suggesting that α-synuclein may also be an unspecific marker of neurodegeneration [193–196]. In addition, α-synuclein is highly expressed in red blood cells. Therefore, blood contamination during sample collection may further limit the diagnostic value [197,198].

4.9. AD biomarkers in blood

The most successful body fluid for finding biomarkers for AD has undoubtedly been CSF, probably because its proximity to the brain and the pathologic processes of interest. However, for practical reasons, blood-based biomarkers would be superior in clinical routine. Despite much research in this field, there is still no established blood-based biomarker for AD. Brain-derived proteins occur in lower concentrations in blood than in CSF, at least partly because of the blood–brain barrier, which limits the transport of substances between blood vessels and the brain parenchyma, but probably also because of degradation. The analysis of Aβ peptides in blood in AD patients and controls has most frequently shown similar levels [199]. Recent approaches using techniques where several biomarkers are analyzed simultaneously have identified promising biomarkers [200,201], but the results have unfortunately been hard to replicate [202,203].

5. STANDARDIZATION EFFORTS

The clinical utility of CSF tests for T-tau, P-tau, and Aβ42 is clear and their importance for selecting patients in predementia stages of AD for clinical trials of disease-modifying drug candidates is undisputable [204]. However, most of the commercially available assays for these biomarkers are still research grade, and there are no common calibrators or certified reference measurement systems at hand [205]. This leads to bias in the biomarker measurements across different assay platforms. Furthermore, even when the same assay is used, variation in biomarker measurements between laboratories is high, which can be seen in multicenter comparisons of measurements [206], including the Alzheimer's Association quality control (QC) program for CSF biomarkers [207,208]. This program includes around 90 participants around the globe and shows that the interlaboratory coefficients of variation (CVs) for commercially available tau and Aβ assays are between 20% and 30%, whereas intralaboratory studies show that CVs of $<$10% should be feasible.

Important preanalytical sources of variation for the most difficult AD biomarker, Aβ42, are storage tube type (polypropylene tubes are recommended, but different brands may show different analyte adsorption [209]), sample aliquot volume [210], and blood contamination [211]. Analytical sources of variation include the composition of the diluent buffer; low concentrations of detergent increase the measured Aβ42 concentration,

which has to be standardized [211]. Several additional factors may be important in an assay-specific manner and close adherence to kit inserts is recommended, as is participation in the Alzheimer's Association QC program and other interlaboratory comparison programs to ensure that proper laboratory procedures are in place.

To solve bias and variation problems, a number of standardization efforts have been initiated, all aimed at facilitating the development of standard operating procedures for preanalytical sample handling and assay procedures, as well as reference methods and materials for the key analytes. These initiatives include the Alzheimer's Association Global Consortium for Biomarker Standardization (GCBS) and the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) Working Group for CSF Proteins [212]. Standard operating procedures for CSF sampling and storage have been published [82] and a selected reaction monitoring mass spectrometry-based method for Aβ1–42 has been described [213]. Strong collaborative efforts within GCBS are underway and updates are available on http://www.alz.org/research/funding/global_biomarker_consortium.asp.

6. CONCLUDING REMARKS

CSF T-tau, P-tau, and Aβ42 are clinically useful markers of AD pathology. In the absence of definite results from ongoing standardization efforts, it is still possible to upgrade currently available tests for use in clinical laboratory routine. We perform CSF T-tau, P-tau, and Aβ42 measurements by INNOTEST ELISAs twice a week using procedures that are accredited by the Swedish Board for Accreditation and Conformity Assessment and have managed to measure CSF T-tau and P-tau concentrations in a reasonably stable manner over more than 10 years according to a system of internal QC samples representing clinically relevant low and high concentrations of the analytes. Careful bridging of QC pools and incoming kit lots has been important to maintain and monitor analytical stability. CSF Aβ42 has been more challenging, and recently, we have had to adjust the normal reference limit from <450 to <550 ng/L due to changes in the calibration of the assay. Laboratories that want to establish these analyses in clinical routine are presently encouraged to develop their own reference limits or align to laboratories that have already done so. Moreover, enrollment in an external QC program, such as the Alzheimer's Association QC program, is strongly recommended. In a 10-year perspective, the hope is that reference methods and materials for CSF T-tau, P-tau, and Aβ42, as well as automated assays,

will be in place so that globally applicable reference ranges and decision limits for these markers can be employed, which will make it easier to compare studies and to develop detailed diagnostic algorithms. It is also reasonable to hope for the establishment of novel biomarkers for molecular pathologies that may be seen in important differential diagnoses to AD. As a closing comment, we wish to emphasize that the future of the CSF biomarker field depends to a very large extent on whether disease-modifying therapies against specific molecular changes in different neurodegenerative conditions eventually are blessed with clinical success.

ACKNOWLEDGMENTS

Work in the authors' laboratories is supported by the Swedish Research Council, Alzheimer's Association, Swedish State Support for Clinical Research, and the Wolfson Foundation. We wish to thank colleagues and patients and their families for generating the extensive literature that was reviewed here.

REFERENCES

[1] A. Alzheimer, R.A. Stelzmann, H.N. Schnitzlein, F.R. Murtagh, An English translation of Alzheimer's 1907 paper, "Uber eine eigenartige Erkankung der Hirnrinde" Clin. Anat. 8 (6) (1995) 429–431.
[2] K. Blennow, M.J. De Leon, H. Zetterberg, Alzheimer's disease, Lancet 368 (9533) (2006) 387–403.
[3] B.E. Tomlinson, G. Blessed, M. Roth, Observations on the brains of demented old people, J. Neurol. Sci. 11 (3) (1970) 205–242.
[4] R. Katzman, Alzheimer's disease, N. Engl. J. Med. 314 (15) (1986) 964–973.
[5] M. Roth, B.E. Tomlinson, G. Blessed, Correlation between scores for dementia and counts of 'senile plaques' in cerebral grey matter of elderly subjects, Nature 209 (5018) (1966) 109–110.
[6] G.G. Glenner, C.W. Wong, Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein, Biochem. Biophys. Res. Commun. 120 (3) (1984) 885–890.
[7] C.L. Masters, G. Simms, N.A. Weinman, G. Multhaup, B.L. Mcdonald, K. Beyreuther, Amyloid plaque core protein in Alzheimer disease and Down syndrome, Proc. Natl. Acad. Sci. U.S.A. 82 (12) (1985) 4245–4249.
[8] E. Portelius, N. Bogdanovic, M.K. Gustavsson, et al., Mass spectrometric characterization of brain amyloid beta isoform signatures in familial and sporadic Alzheimer's disease, Acta Neuropathol. 120 (2) (2010) 185–193.
[9] I. Grundke-Iqbal, K. Iqbal, Y.C. Tung, M. Quinlan, H.M. Wisniewski, L.I. Binder, Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology, Proc. Natl. Acad. Sci. U.S.A. 83 (13) (1986) 4913–4917.
[10] N. Nukina, Y. Ihara, One of the antigenic determinants of paired helical filaments is related to tau protein, J. Biochem. 99 (5) (1986) 1541–1544.
[11] E.M. Mandelkow, E. Mandelkow, Biochemistry and cell biology of tau protein in neurofibrillary degeneration, Cold Spring Harb. Perspect. Med. 2 (7) (2012) a006247.

[12] J. Kang, H.G. Lemaire, A. Unterbeck, et al., The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor, Nature 325 (6106) (1987) 733–736.
[13] D. Goldgaber, M.I. Lerman, O.W. Mcbride, U. Saffiotti, D.C. Gajdusek, Characterization and chromosomal localization of a cDNA encoding brain amyloid of Alzheimer's disease, Science 235 (4791) (1987) 877–880.
[14] E.R. Popovitch, H.M. Wisniewski, M. Barcikowska, et al., Alzheimer neuropathology in non-Down's syndrome mentally retarded adults, Acta Neuropathol. 80 (4) (1990) 362–367.
[15] W.E. Van Nostrand, S.L. Wagner, M. Suzuki, et al., Protease nexin-II, a potent antichymotrypsin, shows identity to amyloid beta-protein precursor, Nature 341 (6242) (1989) 546–549.
[16] N.N. Nalivaeva, A.J. Turner, The amyloid precursor protein: a biochemical enigma in brain development, function and disease, FEBS Lett. 587 (13) (2013) 2046–2054.
[17] M. Mullan, F. Crawford, K. Axelman, et al., A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of beta-amyloid, Nat. Genet. 1 (5) (1992) 345–347.
[18] C.M. Van Duijn, L. Hendriks, M. Cruts, J.A. Hardy, A. Hofman, C. Van Broeckhoven, Amyloid precursor protein gene mutation in early-onset Alzheimer's disease, Lancet 337 (8747) (1991) 978.
[19] M.C. Chartier-Harlin, F. Crawford, H. Houlden, et al., Early-onset Alzheimer's disease caused by mutations at codon 717 of the beta-amyloid precursor protein gene, Nature 353 (6347) (1991) 844–846.
[20] D.J. Selkoe, Alzheimer's disease: genes, proteins, and therapy, Physiol. Rev. 81 (2) (2001) 741–766.
[21] L. Chavez-Gutierrez, L. Bammens, I. Benilova, et al., The mechanism of gamma-secretase dysfunction in familial Alzheimer disease, EMBO J. 31 (10) (2012) 2261–2274.
[22] D.M. Walsh, D.J. Selkoe, A beta oligomers—a decade of discovery, J. Neurochem. 101 (5) (2007) 1172–1184.
[23] J.A. Hardy, G.A. Higgins, Alzheimer's disease: the amyloid cascade hypothesis, Science 256 (5054) (1992) 184–185.
[24] J. Hardy, The amyloid hypothesis for Alzheimer's disease: a critical reappraisal, J. Neurochem. 110 (4) (2009) 1129–1134.
[25] K. Blennow, A. Wallin, Clinical heterogeneity of probable Alzheimer's disease, J. Geriatr. Psychiatry Neurol. 5 (2) (1992) 106–113.
[26] T. Jonsson, J.K. Atwal, S. Steinberg, et al., A mutation in APP protects against Alzheimer's disease and age-related cognitive decline, Nature 488 (7409) (2012) 96–99.
[27] N.L. Pedersen, M. Gatz, S. Berg, B. Johansson, How heritable is Alzheimer's disease late in life? Findings from Swedish twins, Ann. Neurol. 55 (2) (2004) 180–185.
[28] M. Gatz, C.A. Reynolds, L. Fratiglioni, et al., Role of genes and environments for explaining Alzheimer disease, Arch. Gen. Psychiatry 63 (2) (2006) 168–174.
[29] D.M. Holtzman, J. Herz, G. Bu, Apolipoprotein E and apolipoprotein E receptors: normal biology and roles in Alzheimer disease, Cold Spring Harb. Perspect. Med. 2 (3) (2012) a006312.
[30] L. Bertram, R.E. Tanzi, The genetics of Alzheimer's disease, Prog. Mol. Biol. Transl. Sci. 107 (2012) 79–100.
[31] L. Jones, P.A. Holmans, M.L. Hamshere, et al., Genetic evidence implicates the immune system and cholesterol metabolism in the aetiology of Alzheimer's disease, PLoS One 5 (11) (2010) e13950.

[32] R. Guerreiro, A. Wojtas, J. Bras, et al., TREM2 variants in Alzheimer's disease, N. Engl. J. Med. 368 (2) (2013) 117–127.
[33] T. Jonsson, H. Stefansson, S. Steinberg, et al., Variant of TREM2 associated with the risk of Alzheimer's disease, N. Engl. J. Med. 368 (2) (2013) 107–116.
[34] A.M. Fjell, L. Mcevoy, D. Holland, A.M. Dale, K.B. Walhovd, Brain changes in older adults at very low risk for Alzheimer's disease, J. Neurosci. 33 (19) (2013) 8237–8242.
[35] D.R. Thal, U. Rub, M. Orantes, H. Braak, Phases of A beta-deposition in the human brain and its relevance for the development of AD, Neurology 58 (12) (2002) 1791–1800.
[36] H. Braak, H. Zetterberg, K. Del Tredici, K. Blennow, Intraneuronal tau aggregation precedes diffuse plaque deposition, but amyloid-beta changes occur before increases of tau in cerebrospinal fluid, Acta Neuropathol. 126 (5) (2013) 631–641.
[37] P.T. Nelson, I. Alafuzoff, E.H. Bigio, et al., Correlation of Alzheimer disease neuropathologic changes with cognitive status: a review of the literature, J. Neuropathol. Exp. Neurol. 71 (5) (2012) 362–381.
[38] A. Wojtas, K.A. Heggeli, N. Finch, et al., C9ORF72 repeat expansions and other FTD gene mutations in a clinical AD patient series from Mayo Clinic, Am. J. Neurodegener. Dis. 1 (1) (2012) 107–118.
[39] S.C. Jin, P. Pastor, B. Cooper, et al., Pooled-DNA sequencing identifies novel causative variants in PSEN1, GRN and MAPT in a clinical early-onset and familial Alzheimer's disease Ibero-American cohort, Alzheimers Res. Ther. 4 (4) (2012) 34.
[40] A. Alzheimer, H. Forstl, R. Levy, On certain peculiar diseases of old age, Hist. Psychiatry 2 (5 Pt. 1) (1991) 71–101.
[41] L.A. Hansen, E. Masliah, D. Galasko, R.D. Terry, Plaque-only Alzheimer disease is usually the Lewy body variant, and vice versa, J. Neuropathol. Exp. Neurol. 52 (6) (1993) 648–654.
[42] H. Braak, K. Del Tredici, The pathological process underlying Alzheimer's disease in individuals under thirty, Acta Neuropathol. 121 (2) (2011) 171–181.
[43] C.G. Gottfries, I. Gottfries, B.E. Roos, Homovanillic acid and 5-hydroxyindoleacetic acid in the cerebrospinal fluid of patients with senile dementia, presenile dementia and parkinsonism, J. Neurochem. 16 (9) (1969) 1341–1345.
[44] C.G. Gottfries, I. Gottfries, B.E. Roos, Homovanillic acid and 5-hydroxyindoleacetic acid in cerebrospinal fluid related to rated mental and motor impairment in senile and presenile dementia, Acta Psychiatr. Scand. 46 (2) (1970) 99–105.
[45] C.G. Gottfries, A. Kjallquist, U. Ponten, B.E. Roos, G. Sundbarg, Cerebrospinal fluid pH and monoamine and glucolytic metabolites in Alzheimer's disease, Br. J. Psychiatry 124 (1974) 280–287.
[46] G.G. Glenner, C.W. Wong, Alzheimer's disease and Down's syndrome: sharing of a unique cerebrovascular amyloid fibril protein, Biochem. Biophys. Res. Commun. 122 (3) (1984) 1131–1135.
[47] C. Haass, M.G. Schlossmacher, A.Y. Hung, et al., Amyloid beta-peptide is produced by cultured cells during normal metabolism, Nature 359 (6393) (1992) 322–325.
[48] E. Portelius, E. Price, G. Brinkmalm, et al., A novel pathway for amyloid precursor protein processing, Neurobiol. Aging 32 (6) (2011) 1090–1098.
[49] R. Motter, C. Vigo-Pelfrey, D. Kholodenko, et al., Reduction of beta-amyloid peptide42 in the cerebrospinal fluid of patients with Alzheimer's disease, Ann. Neurol. 38 (4) (1995) 643–648.
[50] C. Rosen, O. Hansson, K. Blennow, H. Zetterberg, Fluid biomarkers in Alzheimer's disease—current concepts, Mol. Neurodegener. 8 (2013) 20.
[51] D. Strozyk, K. Blennow, L.R. White, L.J. Launer, CSF Abeta 42 levels correlate with amyloid-neuropathology in a population-based autopsy study, Neurology 60 (4) (2003) 652–656.

[52] A.M. Fagan, M.A. Mintun, R.H. Mach, et al., Inverse relation between in vivo amyloid imaging load and cerebrospinal fluid Abeta42 in humans, Ann. Neurol. 59 (3) (2006) 512–519.
[53] A. Forsberg, H. Engler, O. Almkvist, et al., PET imaging of amyloid deposition in patients with mild cognitive impairment, Neurobiol. Aging 29 (10) (2008) 1456–1465.
[54] T.T. Seppala, O. Nerg, A.M. Koivisto, et al., CSF biomarkers for Alzheimer disease correlate with cortical brain biopsy findings, Neurology 78 (20) (2012) 1568–1575.
[55] N. Andreasen, L. Minthon, E. Vanmechelen, et al., Cerebrospinal fluid tau and Abeta42 as predictors of development of Alzheimer's disease in patients with mild cognitive impairment, Neurosci. Lett. 273 (1) (1999) 5–8.
[56] O. Hansson, H. Zetterberg, P. Buchhave, E. Londos, K. Blennow, L. Minthon, Association between CSF biomarkers and incipient Alzheimer's disease in patients with mild cognitive impairment: a follow-up study, Lancet Neurol. 5 (3) (2006) 228–234.
[57] L.M. Shaw, H. Vanderstichele, M. Knapik-Czajka, et al., Cerebrospinal fluid biomarker signature in Alzheimer's disease neuroimaging initiative subjects, Ann. Neurol. 65 (4) (2009) 403–413.
[58] P.J. Visser, F. Verhey, D.L. Knol, et al., Prevalence and prognostic value of CSF markers of Alzheimer's disease pathology in patients with subjective cognitive impairment or mild cognitive impairment in the DESCRIPA study: a prospective cohort study, Lancet Neurol. 8 (7) (2009) 619–627.
[59] P. Buchhave, L. Minthon, H. Zetterberg, A.K. Wallin, K. Blennow, O. Hansson, Cerebrospinal fluid levels of beta-amyloid 1-42, but not of Tau, are fully changed already 5 to 10 years before the onset of Alzheimer dementia, Arch. Gen. Psychiatry 69 (1) (2012) 98–106.
[60] I. Skoog, P. Davidsson, O. Aevarsson, H. Vanderstichele, E. Vanmechelen, K. Blennow, Cerebrospinal fluid beta-amyloid 42 is reduced before the onset of sporadic dementia: a population-based study in 85-year-olds, Dement. Geriatr. Cogn. Disord. 15 (3) (2003) 169–176.
[61] A.M. Fagan, D. Head, A.R. Shah, et al., Decreased cerebrospinal fluid Abeta(42) correlates with brain atrophy in cognitively normal elderly, Ann. Neurol. 65 (2) (2009) 176–183.
[62] D.R. Gustafson, I. Skoog, L. Rosengren, H. Zetterberg, K. Blennow, Cerebrospinal fluid beta-amyloid 1-42 concentration may predict cognitive decline in older women, J. Neurol. Neurosurg. Psychiatry 78 (5) (2007) 461–464.
[63] M. Sjogren, M. Gisslen, E. Vanmechelen, K. Blennow, Low cerebrospinal fluid beta-amyloid 42 in patients with acute bacterial meningitis and normalization after treatment, Neurosci. Lett. 314 (1–2) (2001) 33–36.
[64] N. Mattsson, M. Axelsson, S. Haghighi, et al., Reduced cerebrospinal fluid BACE1 activity in multiple sclerosis, Mult. Scler. 15 (2009) 448–454.
[65] M. Gisslen, J. Krut, U. Andreasson, et al., Amyloid and tau cerebrospinal fluid biomarkers in HIV infection, BMC Neurol. 9 (2009) 63.
[66] N. Mattsson, D. Bremell, R. Anckarsater, et al., Neuroinflammation in Lyme neuroborreliosis affects amyloid metabolism, BMC Neurol. 10 (2010) 51.
[67] N.S. Schoonenboom, C. Mulder, G.J. Van Kamp, et al., Amyloid beta 38, 40, and 42 species in cerebrospinal fluid: more of the same? Ann. Neurol. 58 (1) (2005) 139–142.
[68] E. Portelius, H. Zetterberg, U. Andreasson, et al., An Alzheimer's disease-specific beta-amyloid fragment signature in cerebrospinal fluid, Neurosci. Lett. 409 (3) (2006) 215–219.
[69] N. Mattsson, E. Portelius, S. Rolstad, et al., Longitudinal cerebrospinal fluid biomarkers over four years in mild cognitive impairment, J. Alzheimers Dis. 30 (4) (2012) 767–778.

[70] M. Vandermeeren, M. Mercken, E. Vanmechelen, et al., Detection of tau proteins in normal and Alzheimer's disease cerebrospinal fluid with a sensitive sandwich enzyme-linked immunosorbent assay, J. Neurochem. 61 (5) (1993) 1828–1834.
[71] K. Blennow, A. Wallin, H. Agren, C. Spenger, J. Siegfried, E. Vanmechelen, Tau protein in cerebrospinal fluid: a biochemical marker for axonal degeneration in Alzheimer disease? Mol. Chem. Neuropathol. 26 (3) (1995) 231–245.
[72] L. Wang, A.M. Fagan, A.R. Shah, et al., Cerebrospinal fluid proteins predict longitudinal hippocampal degeneration in early-stage dementia of the Alzheimer type, Alzheimer Dis. Assoc. Disord. 26 (4) (2012) 314–321.
[73] L. Glodzik, L. Mosconi, W. Tsui, et al., Alzheimer's disease markers, hypertension, and gray matter damage in normal elderly, Neurobiol. Aging 33 (7) (2012) 1215–1227.
[74] J.Q. Trojanowski, T. Schuck, M.L. Schmidt, V.M. Lee, Distribution of tau proteins in the normal human central and peripheral nervous system, J. Histochem. Cytochem. 37 (2) (1989) 209–215.
[75] C. Hesse, L. Rosengren, N. Andreasen, et al., Transient increase in total tau but not phospho-tau in human cerebrospinal fluid after acute stroke, Neurosci. Lett. 297 (3) (2001) 187–190.
[76] H. Zetterberg, M.A. Hietala, M. Jonsson, et al., Neurochemical aftermath of amateur boxing, Arch. Neurol. 63 (9) (2006) 1277–1280.
[77] A.K. Wallin, K. Blennow, H. Zetterberg, E. Londos, L. Minthon, O. Hansson, CSF biomarkers predict a more malignant outcome in Alzheimer disease, Neurology 74 (19) (2010) 1531–1537.
[78] P. Sanchez-Juan, R. Sanchez-Valle, A. Green, et al., Influence of timing on CSF tests value for Creutzfeldt-Jakob disease diagnosis, J. Neurol. 254 (7) (2007) 901–906.
[79] S. Saman, W. Kim, M. Raya, et al., Exosome-associated tau is secreted in tauopathy models and is selectively phosphorylated in cerebrospinal fluid in early Alzheimer disease, J. Biol. Chem. 287 (6) (2012) 3842–3849.
[80] L.F. Maia, S.A. Kaeser, J. Reichwald, et al., Changes in amyloid-beta and tau in the cerebrospinal fluid of transgenic mice overexpressing amyloid precursor protein, Sci. Transl. Med. 5 (194) (2013) 194re192.
[81] J.E. Meredith Jr., S. Sankaranarayanan, V. Guss, et al., Characterization of novel CSF Tau and ptau biomarkers for Alzheimer's disease, PLoS One 8 (10) (2013) e76523.
[82] K. Blennow, H. Hampel, M. Weiner, H. Zetterberg, Cerebrospinal fluid and plasma biomarkers in Alzheimer disease, Nat. Rev. Neurol. 6 (3) (2010) 131–144.
[83] K. Uryu, X.H. Chen, D. Martinez, et al., Multiple proteins implicated in neurodegenerative diseases accumulate in axons after brain trauma in humans, Exp. Neurol. 208 (2) (2007) 185–192.
[84] C.M. Wischik, M. Novak, H.C. Thogersen, et al., Isolation of a fragment of tau derived from the core of the paired helical filament of Alzheimer disease, Proc. Natl. Acad. Sci. U.S.A. 85 (12) (1988) 4506–4510.
[85] H. Hampel, K. Buerger, R. Zinkowski, et al., Measurement of phosphorylated tau epitopes in the differential diagnosis of Alzheimer disease: a comparative cerebrospinal fluid study, Arch. Gen. Psychiatry 61 (1) (2004) 95–102.
[86] K. Buerger, M. Ewers, T. Pirttila, et al., CSF phosphorylated tau protein correlates with neocortical neurofibrillary pathology in Alzheimer's disease, Brain 129 (Pt. 11) (2006) 3035–3041.
[87] N. Mattsson, K. Savman, G. Osterlundh, K. Blennow, H. Zetterberg, Converging molecular pathways in human neural development and degeneration, Neurosci. Res. 66 (2010) 330–332.
[88] A. Grahn, L. Hagberg, S. Nilsson, K. Blennow, H. Zetterberg, M. Studahl, Cerebrospinal fluid biomarkers in patients with varicella-zoster virus CNS infections, J. Neurol. 260 (7) (2013) 1813–1821.

[89] D. Kondziella, H. Zetterberg, Hyperphosphorylation of tau protein in superficial CNS siderosis, J. Neurol. Sci. 273 (1–2) (2008) 130–132.
[90] T. Ikeda, D. Noto, M. Noguchi-Shinohara, et al., CSF tau protein is a useful marker for effective treatment of superficial siderosis of the central nervous system: two case reports, Clin. Neurol. Neurosurg. 112 (1) (2010) 62–64.
[91] C.T. Williams, B.M. Barnes, M. Richter, C.L. Buck, Hibernation and circadian rhythms of body temperature in free-living Arctic ground squirrels, Physiol. Biochem. Zool. 85 (4) (2012) 397–404.
[92] W. Hartig, J. Stieler, A.S. Boerema, et al., Hibernation model of tau phosphorylation in hamsters: selective vulnerability of cholinergic basal forebrain neurons—implications for Alzheimer's disease, Eur. J. Neurosci. 25 (1) (2007) 69–80.
[93] P. Johansson, N. Mattsson, O. Hansson, et al., Cerebrospinal fluid biomarkers for Alzheimer's disease: diagnostic performance in a homogeneous mono-center population, J. Alzheimers Dis. 24 (3) (2011) 537–546.
[94] N. Mattsson, H. Zetterberg, O. Hansson, et al., CSF biomarkers and incipient Alzheimer disease in patients with mild cognitive impairment, JAMA 302 (4) (2009) 385–393.
[95] T.P. Baumann, H. Duyar, M. Sollberger, et al., CSF-tau and CSF-Abeta(1-42) in posterior cortical atrophy, Dement. Geriatr. Cogn. Disord. 29 (6) (2010) 530–533.
[96] J. Seguin, M. Formaglio, A. Perret-Liaudet, et al., CSF biomarkers in posterior cortical atrophy, Neurology 76 (21) (2011) 1782–1788.
[97] M. Bibl, B. Mollenhauer, P. Lewczuk, et al., Cerebrospinal fluid tau, p-tau 181 and amyloid-beta38/40/42 in frontotemporal dementias and primary progressive aphasias, Dement. Geriatr. Cogn. Disord. 31 (1) (2011) 37–44.
[98] B. Borroni, E. Premi, C. Agosti, et al., CSF Alzheimer's disease-like pattern in corticobasal syndrome: evidence for a distinct disorder, J. Neurol. Neurosurg. Psychiatry 82 (8) (2011) 834–838.
[99] J.B. Toledo, S.X. Xie, J.Q. Trojanowski, L.M. Shaw, Longitudinal change in CSF tau and Abeta biomarkers for up to 48 months in ADNI, Acta Neuropathol. 126 (5) (2013) 659–670.
[100] N. Mattsson, P. Insel, R. Nosheny, et al., CSF protein biomarkers predicting longitudinal reduction of CSF beta-amyloid42 in cognitively healthy elders, Transl. Psychiatry 3 (2013) e293.
[101] A. Moghekar, S. Li, Y. Lu, et al., CSF biomarker changes precede symptom onset of mild cognitive impairment, Neurology 81 (2013) 1753–1758.
[102] H. Zetterberg, M. Pedersen, K. Lind, et al., Intra-individual stability of CSF biomarkers for Alzheimer's disease over two years, J. Alzheimers Dis. 12 (3) (2007) 255–260.
[103] K. Blennow, H. Zetterberg, L. Minthon, et al., Longitudinal stability of CSF biomarkers in Alzheimer's disease, Neurosci. Lett. 419 (1) (2007) 18–22.
[104] N. Mattsson, L. Rajendran, H. Zetterberg, et al., BACE1 inhibition induces a specific cerebrospinal fluid beta-amyloid pattern that identifies drug effects in the central nervous system, PLoS One 7 (2) (2012) e31084.
[105] L. Lannfelt, K. Blennow, H. Zetterberg, et al., Safety, efficacy, and biomarker findings of PBT2 in targeting Abeta as a modifying therapy for Alzheimer's disease: a phase IIa, double-blind, randomised, placebo-controlled trial, Lancet Neurol. 7 (9) (2008) 779–786.
[106] P.C. May, R.A. Dean, S.L. Lowe, et al., Robust central reduction of amyloid-beta in humans with an orally available, non-peptidic beta-secretase inhibitor, J. Neurosci. 31 (46) (2011) 16507–16516.
[107] E. Portelius, R.A. Dean, M.K. Gustavsson, et al., A novel Abeta isoform pattern in CSF reflects gamma-secretase inhibition in Alzheimer disease, Alzheimers Res. Ther. 2 (2) (2010) 7.

[108] S. Gilman, M. Koller, R.S. Black, et al., Clinical effects of Abeta immunization (AN1792) in patients with AD in an interrupted trial, Neurology 64 (9) (2005) 1553–1562.
[109] K. Blennow, H. Zetterberg, J.O. Rinne, et al., Effect of immunotherapy with bapineuzumab on cerebrospinal fluid biomarker levels in patients with mild to moderate Alzheimer disease, Arch. Neurol. 69 (8) (2012) 1002–1010.
[110] J.M. Schott, J.D. Warren, Alzheimer's disease: mimics and chameleons, Pract. Neurol. 12 (6) (2012) 358–366.
[111] Y.S. Shim, C.M. Roe, V.D. Buckles, J.C. Morris, Clinicopathologic study of Alzheimer's disease: Alzheimer mimics, J. Alzheimers Dis. 35 (4) (2013) 799–811.
[112] U. Andreasson, E. Portelius, M.E. Andersson, K. Blennow, H. Zetterberg, Aspects of beta-amyloid as a biomarker for Alzheimer's disease, Biomark. Med 1 (1) (2007) 59–78.
[113] H. Fukumoto, B.S. Cheung, B.T. Hyman, M.C. Irizarry, Beta-secretase protein and activity are increased in the neocortex in Alzheimer disease, Arch. Neurol. 59 (9) (2002) 1381–1389.
[114] R.M. Holsinger, J.S. Lee, A. Boyd, C.L. Masters, S.J. Collins, CSF BACE1 activity is increased in CJD and Alzheimer disease versus [corrected] other dementias, Neurology 67 (4) (2006) 710–712.
[115] R.M. Holsinger, C.A. Mclean, S.J. Collins, C.L. Masters, G. Evin, Increased beta-secretase activity in cerebrospinal fluid of Alzheimer's disease subjects, Ann. Neurol. 55 (6) (2004) 898–899.
[116] H. Zetterberg, U. Andreasson, O. Hansson, et al., Elevated cerebrospinal fluid BACE1 activity in incipient Alzheimer disease, Arch. Neurol. 65 (8) (2008) 1102–1107.
[117] S.D. Mulder, W.M. Van Der Flier, J.H. Verheijen, et al., BACE1 activity in cerebrospinal fluid and its relation to markers of AD pathology, J. Alzheimers Dis. 20 (1) (2010) 253–260.
[118] Z. Zhong, M. Ewers, S. Teipel, et al., Levels of beta-secretase (BACE1) in cerebrospinal fluid as a predictor of risk in mild cognitive impairment, Arch. Gen. Psychiatry 64 (6) (2007) 718–726.
[119] C. Rosen, U. Andreasson, N. Mattsson, et al., Cerebrospinal fluid profiles of amyloid beta-related biomarkers in Alzheimer's disease, Neuromolecular Med. 14 (1) (2012) 65–73.
[120] A. Olsson, K. Hoglund, M. Sjogren, et al., Measurement of alpha- and beta-secretase cleaved amyloid precursor protein in cerebrospinal fluid from Alzheimer patients, Exp. Neurol. 183 (1) (2003) 74–80.
[121] R. Perneczky, A. Tsolakidou, A. Arnold, et al., CSF soluble amyloid precursor proteins in the diagnosis of incipient Alzheimer disease, Neurology 77 (1) (2011) 35–38.
[122] P. Lewczuk, H. Kamrowski-Kruck, O. Peters, et al., Soluble amyloid precursor proteins in the cerebrospinal fluid as novel potential biomarkers of Alzheimer's disease: a multicenter study, Mol. Psychiatry 15 (2) (2010) 138–145.
[123] P. Lewczuk, J. Popp, N. Lelental, et al., Cerebrospinal fluid soluble amyloid-beta protein precursor as a potential novel biomarkers of Alzheimer's disease, J. Alzheimers Dis. 28 (1) (2012) 119–125.
[124] A. Gabelle, S. Roche, C. Geny, et al., Correlations between soluble alpha/beta forms of amyloid precursor protein and Abeta38, 40, and 42 in human cerebrospinal fluid, Brain Res. 1357 (2010) 175–183.
[125] R.J. Castellani, M.A. Smith, Compounding artefacts with uncertainty, and an amyloid cascade hypothesis that is 'too big to fail', J. Pathol. 224 (2) (2011) 147–152.
[126] D.M. Walsh, I. Klyubin, J.V. Fadeeva, et al., Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo, Nature 416 (6880) (2002) 535–539.

[127] H. Zempel, E. Thies, E. Mandelkow, E.M. Mandelkow, Abeta oligomers cause localized Ca(2+) elevation, missorting of endogenous tau into dendrites, tau phosphorylation, and destruction of microtubules and spines, J. Neurosci. 30 (36) (2010) 11938–11950.
[128] M. Jin, N. Shepardson, T. Yang, G. Chen, D. Walsh, D.J. Selkoe, Soluble amyloid beta-protein dimers isolated from Alzheimer cortex directly induce Tau hyperphosphorylation and neuritic degeneration, Proc. Natl. Acad. Sci. U.S.A. 108 (14) (2011) 5819–5824.
[129] F.G. De Felice, D. Wu, M.P. Lambert, et al., Alzheimer's disease-type neuronal tau hyperphosphorylation induced by A beta oligomers, Neurobiol. Aging 29 (9) (2008) 1334–1347.
[130] K.A. Bruggink, W. Jongbloed, E.A. Biemans, et al., Amyloid-beta oligomer detection by ELISA in cerebrospinal fluid and brain tissue, Anal. Biochem. 433 (2) (2013) 112–120.
[131] G.M. Shankar, S. Li, T.H. Mehta, et al., Amyloid-beta protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory, Nat. Med. 14 (8) (2008) 837–842.
[132] C.M. Gao, A.Y. Yam, X. Wang, et al., Abeta40 oligomers identified as a potential biomarker for the diagnosis of Alzheimer's disease, PLoS One 5 (12) (2010) e15725.
[133] H. Fukumoto, T. Tokuda, T. Kasai, et al., High-molecular-weight {beta}-amyloid oligomers are elevated in cerebrospinal fluid of Alzheimer patients, FASEB J. 24 (2010) 2716–2726.
[134] D.G. Georganopoulou, L. Chang, J.M. Nam, et al., Nanoparticle-based detection in cerebral spinal fluid of a soluble pathogenic biomarker for Alzheimer's disease, Proc. Natl. Acad. Sci. U.S.A. 102 (7) (2005) 2273–2276.
[135] M. Pitschke, R. Prior, M. Haupt, D. Riesner, Detection of single amyloid beta-protein aggregates in the cerebrospinal fluid of Alzheimer's patients by fluorescence correlation spectroscopy, Nat. Med. 4 (7) (1998) 832–834.
[136] M. Holtta, O. Hansson, U. Andreasson, et al., Evaluating amyloid-beta oligomers in cerebrospinal fluid as a biomarker for Alzheimer's disease, PLoS One 8 (6) (2013) e66381.
[137] M. Handoko, M. Grant, M. Kuskowski, et al., Correlation of specific amyloid-beta oligomers with tau in cerebrospinal fluid from cognitively normal older adults, JAMA Neurol. 70 (5) (2013) 594–599.
[138] A.N. Santos, S. Torkler, D. Nowak, et al., Detection of amyloid-beta oligomers in human cerebrospinal fluid by flow cytometry and fluorescence resonance energy transfer, J. Alzheimers Dis. 11 (1) (2007) 117–125.
[139] K. Blennow, A. Wallin, P. Fredman, I. Karlsson, C.G. Gottfries, L. Svennerholm, Blood-brain barrier disturbance in patients with Alzheimer's disease is related to vascular factors, Acta Neurol. Scand. 81 (4) (1990) 323–326.
[140] A. Wallin, K. Blennow, L. Rosengren, Cerebrospinal fluid markers of pathogenetic processes in vascular dementia, with special reference to the subcortical subtype, Alzheimer Dis. Assoc. Disord. 13 (Suppl. 3) (1999) S102–S105.
[141] H. Tumani, G. Nolker, H. Reiber, Relevance of cerebrospinal fluid variables for early diagnosis of neuroborreliosis, Neurology 45 (9) (1995) 1663–1670.
[142] S. Chalbot, H. Zetterberg, K. Blennow, T. Fladby, I. Grundke-Iqbal, K. Iqbal, Cerebrospinal fluid secretory Ca2+-dependent phospholipase A2 activity: a biomarker of blood-cerebrospinal fluid barrier permeability, Neurosci. Lett. 478 (3) (2010) 179–183.
[143] H. Zetterberg, U. Andreasson, K. Blennow, CSF antithrombin III and disruption of the blood-brain barrier, J. Clin. Oncol. 27 (13) (2009) 2302–2303, author reply 2303–2304.

[144] L.E. Rosengren, J.E. Karlsson, M. Sjogren, K. Blennow, A. Wallin, Neurofilament protein levels in CSF are increased in dementia, Neurology 52 (5) (1999) 1090–1093.
[145] A. Agren-Wilsson, A. Lekman, W. Sjoberg, et al., CSF biomarkers in the evaluation of idiopathic normal pressure hydrocephalus, Acta Neurol. Scand. 116 (5) (2007) 333–339.
[146] A. Wallin, M. Sjogren, Cerebrospinal fluid cytoskeleton proteins in patients with subcortical white-matter dementia, Mech. Ageing Dev. 122 (16) (2001) 1937–1949.
[147] D. De Jong, R.W. Jansen, Y.A. Pijnenburg, et al., CSF neurofilament proteins in the differential diagnosis of dementia, J. Neurol. Neurosurg. Psychiatry 78 (9) (2007) 936–938.
[148] M. Landqvist Waldo, A. Frizell Santillo, U. Passant, et al., Cerebrospinal fluid neurofilament light chain protein levels in subtypes of frontotemporal dementia, BMC Neurol. 13 (2013) 54.
[149] M. Gisslen, L. Hagberg, B.J. Brew, P. Cinque, R.W. Price, L. Rosengren, Elevated cerebrospinal fluid neurofilament light protein concentrations predict the development of AIDS dementia complex, J. Infect. Dis. 195 (12) (2007) 1774–1778.
[150] T. Skillbäck, H. Zetterberg, K. Blennow, N. Mattsson, CSF biomarkers for Alzheimer's disease and subcortical axonal damage in 5542 clinical samples, Alzheimer's Res. Ther. 5 (2013) 47.
[151] A. Jeppsson, H. Zetterberg, K. Blennow, C. Wikkelso, Idiopathic normal-pressure hydrocephalus: pathophysiology and diagnosis by CSF biomarkers, Neurology 80 (15) (2013) 1385–1392.
[152] R. Tortelli, M. Ruggieri, R. Cortese, et al., Elevated cerebrospinal fluid neurofilament light levels in patients with amyotrophic lateral sclerosis: a possible marker of disease severity and progression, Eur. J. Neurol. 19 (12) (2012) 1561–1567.
[153] L.E. Rosengren, J.E. Karlsson, J.O. Karlsson, L.I. Persson, C. Wikkelso, Patients with amyotrophic lateral sclerosis and other neurodegenerative diseases have increased levels of neurofilament protein in CSF, J. Neurochem. 67 (5) (1996) 2013–2018.
[154] W. Swardfager, K. Lanctot, L. Rothenburg, A. Wong, J. Cappell, N. Herrmann, A meta-analysis of cytokines in Alzheimer's disease, Biol. Psychiatry 68 (2010) 930–941.
[155] C. Comi, M. Carecchio, A. Chiocchetti, et al., Osteopontin is increased in the cerebrospinal fluid of patients with Alzheimer's disease and its levels correlate with cognitive decline, J. Alzheimers Dis. 19 (4) (2010) 1143–1148.
[156] P.J. Naude, C. Nyakas, L.E. Eiden, et al., Lipocalin 2: novel component of proinflammatory signaling in Alzheimer's disease, FASEB J. 26 (7) (2012) 2811–2823.
[157] C. Rosen, N. Mattsson, P.M. Johansson, et al., Discriminatory analysis of biochip-derived protein patterns in CSF and plasma in neurodegenerative diseases, Front. Aging Neurosci. 3 (2011) 1.
[158] K. Blennow, A. Wallin, P. Fredman, C.G. Gottfries, I. Karlsson, L. Svennerholm, Intrathecal synthesis of immunoglobulins in patients with Alzheimer's disease, Eur. Neuropsychopharmacol. 1 (1) (1990) 79–81.
[159] J.D. Morrow, L.J. Roberts, The isoprostanes: unique bioactive products of lipid peroxidation, Prog. Lipid Res. 36 (1) (1997) 1–21.
[160] M. Brys, E. Pirraglia, K. Rich, et al., Prediction and longitudinal study of CSF biomarkers in mild cognitive impairment, Neurobiol. Aging 30 (5) (2009) 682–690.
[161] M.J. De Leon, S. Desanti, R. Zinkowski, et al., Longitudinal CSF and MRI biomarkers improve the diagnosis of mild cognitive impairment, Neurobiol. Aging 27 (3) (2006) 394–401.
[162] M. Grossman, J. Farmer, S. Leight, et al., Cerebrospinal fluid profile in frontotemporal dementia and Alzheimer's disease, Ann. Neurol. 57 (5) (2005) 721–729.
[163] T.J. Montine, M.F. Beal, M.E. Cudkowicz, et al., Increased CSF F2-isoprostane concentration in probable AD, Neurology 52 (3) (1999) 562–565.

[164] T.J. Montine, W.R. Markesbery, J.D. Morrow, L.J. Roberts 2nd., Cerebrospinal fluid F2-isoprostane levels are increased in Alzheimer's disease, Ann. Neurol. 44 (3) (1998) 410–413.
[165] J.M. Ringman, S.G. Younkin, D. Pratico, et al., Biochemical markers in persons with preclinical familial Alzheimer disease, Neurology 71 (2) (2008) 85–92.
[166] F.H. Duits, M.I. Kester, P.G. Scheffer, et al., Increase in cerebrospinal fluid F2-isoprostanes is related to cognitive decline in APOE epsilon4 carriers, J. Alzheimers Dis. 36 (3) (2013) 563–570.
[167] D. Gackowski, R. Rozalski, A. Siomek, et al., Oxidative stress and oxidative DNA damage is characteristic for mixed Alzheimer disease/vascular dementia, J. Neurol. Sci. 266 (1–2) (2008) 57–62.
[168] P. Podlesniy, J. Figueiro-Silva, A. Llado, et al., Low cerebrospinal fluid concentration of mitochondrial DNA in preclinical Alzheimer disease, Ann. Neurol. 74 (2013) 655–668.
[169] G.H. Renkema, R.G. Boot, F.L. Au, et al., Chitotriosidase, a chitinase, and the 39-kDa human cartilage glycoprotein, a chitin-binding lectin, are homologues of family 18 glycosyl hydrolases secreted by human macrophages, Eur. J. Biochem. 251 (1–2) (1998) 504–509.
[170] C.E. Hollak, S. Van Weely, M.H. Van Oers, J.M. Aerts, Marked elevation of plasma chitotriosidase activity. A novel hallmark of Gaucher disease, J. Clin. Invest. 93 (3) (1994) 1288–1292.
[171] M. Watabe-Rudolph, Z. Song, L. Lausser, et al., Chitinase enzyme activity in CSF is a powerful biomarker of Alzheimer disease, Neurology 78 (8) (2012) 569–577.
[172] B.E. Hakala, C. White, A.D. Recklies, Human cartilage gp-39, a major secretory product of articular chondrocytes and synovial cells, is a mammalian member of a chitinase protein family, J. Biol. Chem. 268 (34) (1993) 25803–25810.
[173] R. Craig-Schapiro, R.J. Perrin, C.M. Roe, et al., YKL-40: a novel prognostic fluid biomarker for preclinical Alzheimer's disease, Biol. Psychiatry 68 (10) (2010) 903–912.
[174] B. Olsson, J. Hertze, R. Lautner, et al., Microglial markers are elevated in the prodromal phase of Alzheimer's disease and vascular dementia, J. Alzheimers Dis. 33 (1) (2013) 45–53.
[175] A. Sokolova, M.D. Hill, F. Rahimi, L.A. Warden, G.M. Halliday, C.E. Shepherd, Monocyte chemoattractant protein-1 plays a dominant role in the chronic inflammation observed in Alzheimer's disease, Brain Pathol. 19 (3) (2009) 392–398.
[176] K. Westin, P. Buchhave, H. Nielsen, L. Minthon, S. Janciauskiene, O. Hansson, CCL2 is associated with a faster rate of cognitive decline during early stages of Alzheimer's disease, PLoS One 7 (1) (2012) e30525.
[177] J.D. Correa, D. Starling, A.L. Teixeira, P. Caramelli, T.A. Silva, Chemokines in CSF of Alzheimer's disease patients, Arq. Neuropsiquiatr. 69 (3) (2011) 455–459.
[178] D. Galimberti, N. Schoonenboom, P. Scheltens, et al., Intrathecal chemokine levels in Alzheimer disease and frontotemporal lobar degeneration, Neurology 66 (1) (2006) 146–147.
[179] D. Galimberti, N. Schoonenboom, P. Scheltens, et al., Intrathecal chemokine synthesis in mild cognitive impairment and Alzheimer disease, Arch. Neurol. 63 (4) (2006) 538–543.
[180] N. Mattsson, S. Tabatabaei, P. Johansson, et al., Cerebrospinal fluid microglial markers in Alzheimer's disease: elevated chitotriosidase activity but lack of diagnostic utility, Neuromolecular Med. 13 (2) (2011) 151–159.
[181] I. Blasko, W. Lederer, H. Oberbauer, et al., Measurement of thirteen biological markers in CSF of patients with Alzheimer's disease and other dementias, Dement. Geriatr. Cogn. Disord. 21 (1) (2006) 9–15.

[182] G.N. Yin, H. Jeon, S. Lee, H.W. Lee, J.Y. Cho, K. Suk, Role of soluble CD14 in cerebrospinal fluid as a regulator of glial functions, J. Neurosci. Res. 87 (11) (2009) 2578–2590.
[183] S. Engelborghs, M. De Brabander, J. De Cree, et al., Unchanged levels of interleukins, neopterin, interferon-gamma and tumor necrosis factor-alpha in cerebrospinal fluid of patients with dementia of the Alzheimer type, Neurochem. Int. 34 (6) (1999) 523–530.
[184] R.D. Terry, E. Masliah, D.P. Salmon, et al., Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment, Ann. Neurol. 30 (4) (1991) 572–580.
[185] P. Davidsson, M. Puchades, K. Blennow, Identification of synaptic vesicle, pre- and postsynaptic proteins in human cerebrospinal fluid using liquid-phase isoelectric focusing, Electrophoresis 20 (3) (1999) 431–437.
[186] A. Thorsell, M. Bjerke, J. Gobom, et al., Neurogranin in cerebrospinal fluid as a marker of synaptic degeneration in Alzheimer's disease, Brain Res. 1362 (2010) 13–22.
[187] L. Chang, D.M. Rissin, D.R. Fournier, et al., Single molecule enzyme-linked immunosorbent assays: theoretical considerations, J. Immunol. Methods 378 (1–2) (2012) 102–115.
[188] H.P. Hartung, L. Steinman, D.S. Goodin, et al., Interleukin 17F level and interferon beta response in patients with multiple sclerosis, JAMA Neurol. 70 (8) (2013) 1017–1021.
[189] B. Mollenhauer, O.M. El-Agnaf, K. Marcus, C. Trenkwalder, M.G. Schlossmacher, Quantification of alpha-synuclein in cerebrospinal fluid as a biomarker candidate: review of the literature and considerations for future studies, Biomark. Med 4 (5) (2010) 683–699.
[190] J.L. Guo, D.J. Covell, J.P. Daniels, et al., Distinct alpha-synuclein strains differentially promote tau inclusions in neurons, Cell 154 (1) (2013) 103–117.
[191] O. Pletnikova, N. West, M.K. Lee, et al., Abeta deposition is associated with enhanced cortical alpha-synuclein lesions in Lewy body diseases, Neurobiol. Aging 26 (8) (2005) 1183–1192.
[192] S. Hall, A. Ohrfelt, R. Constantinescu, et al., Accuracy of a panel of 5 cerebrospinal fluid biomarkers in the differential diagnosis of patients with dementia and/or parkinsonian disorders, Arch. Neurol. 69 (2012) 1445–1452.
[193] B. Mollenhauer, J.J. Locascio, W. Schulz-Schaeffer, F. Sixel-Doring, C. Trenkwalder, M.G. Schlossmacher, Alpha-synuclein and tau concentrations in cerebrospinal fluid of patients presenting with parkinsonism: a cohort study, Lancet Neurol. 10 (3) (2011) 230–240.
[194] F. Tateno, R. Sakakibara, T. Kawai, M. Kishi, T. Murano, Alpha-synuclein in the cerebrospinal fluid differentiates synucleinopathies (Parkinson disease, dementia with Lewy bodies, multiple system atrophy) from Alzheimer disease, Alzheimer Dis. Assoc. Disord. 26 (3) (2012) 213–216.
[195] M. Wennstrom, Y. Surova, S. Hall, et al., Low CSF levels of both alpha-synuclein and the alpha-synuclein cleaving enzyme neurosin in patients with synucleinopathy, PLoS One 8 (1) (2013) e53250.
[196] A. Ohrfelt, P. Grognet, N. Andreasen, et al., Cerebrospinal fluid alpha-synuclein in neurodegenerative disorders—a marker of synapse loss? Neurosci. Lett. 450 (3) (2009) 332–335.
[197] R. Barbour, K. Kling, J.P. Anderson, et al., Red blood cells are the major source of alpha-synuclein in blood, Neurodegener. Dis. 5 (2) (2008) 55–59.
[198] Z. Hong, M. Shi, K.A. Chung, et al., DJ-1 and alpha-synuclein in human cerebrospinal fluid as biomarkers of Parkinson's disease, Brain 133 (Pt. 3) (2010) 713–726.
[199] M.C. Irizarry, Biomarkers of Alzheimer disease in plasma, NeuroRx 1 (2) (2004) 226–234.

[200] J.D. Doecke, S.M. Laws, N.G. Faux, et al., Blood-based protein biomarkers for diagnosis of Alzheimer disease, Arch. Neurol. 69 (10) (2012) 1318–1325.
[201] S. Ray, M. Britschgi, C. Herbert, et al., Classification and prediction of clinical Alzheimer's diagnosis based on plasma signaling proteins, Nat. Med. 13 (11) (2007) 1359–1362.
[202] M. Bjorkqvist, M. Ohlsson, L. Minthon, O. Hansson, Evaluation of a previously suggested plasma biomarker panel to identify Alzheimer's disease, PLoS One 7 (1) (2012) e29868.
[203] H.D. Soares, Y. Chen, M. Sabbagh, A. Roher, E. Schrijvers, M. Breteler, Identifying early markers of Alzheimer's disease using quantitative multiplex proteomic immunoassay panels, Ann. N.Y. Acad. Sci. 1180 (2009) 56–67.
[204] H. Hampel, R. Frank, K. Broich, et al., Biomarkers for Alzheimer's disease: academic, industry and regulatory perspectives, Nat. Rev. Drug Discov. 9 (7) (2010) 560–574.
[205] N. Mattsson, I. Zegers, U. Andreasson, et al., Reference measurement procedures for Alzheimer's disease cerebrospinal fluid biomarkers: definitions and approaches with focus on amyloid beta42, Biomark. Med 6 (4) (2012) 409–417.
[206] N.A. Verwey, W.M. Van Der Flier, K. Blennow, et al., A worldwide multicentre comparison of assays for cerebrospinal fluid biomarkers in Alzheimer's disease, Ann. Clin. Biochem. 46 (Pt. 3) (2009) 235–240.
[207] N. Mattsson, U. Andreasson, S. Persson, et al., The Alzheimer's Association external quality control program for cerebrospinal fluid biomarkers, Alzheimers Dement. 7 (4) (2011) 386–395, e6.
[208] N. Mattsson, U. Andreasson, S. Persson, et al., CSF biomarker variability in the Alzheimer's Association quality control program, Alzheimers Dement. 9 (3) (2013) 251–261.
[209] A. Perret-Liaudet, M. Pelpel, Y. Tholance, et al., Risk of Alzheimer's disease biological misdiagnosis linked to cerebrospinal collection tubes, J. Alzheimers Dis. 31 (1) (2012) 13–20.
[210] J. Toombs, R.W. Paterson, M.P. Lunn, et al., Identification of an important potential confound in CSF AD studies: aliquot volume, Clin. Chem. Lab. Med. 51 (2013) 2311–2317.
[211] M. Bjerke, E. Portelius, L. Minthon, et al., Confounding factors influencing amyloid beta concentration in cerebrospinal fluid, Int. J. Alzheimers Dis. 2010 (2010) 1–11.
[212] M.C. Carrillo, K. Blennow, H. Soares, et al., Global standardization measurement of cerebral spinal fluid for Alzheimer's disease: an update from the Alzheimer's Association Global Biomarkers Consortium, Alzheimers Dement. 9 (2) (2013) 137–140.
[213] J. Pannee, E. Portelius, M. Oppermann, et al., A selected reaction monitoring (SRM)-based method for absolute quantification of Abeta38, Abeta40, and Abeta42 in cerebrospinal fluid of Alzheimer's disease patients and healthy controls, J. Alzheimers Dis. 33 (4) (2013) 1021–1032.

CHAPTER SIX

Food Allergy Testing

David J. Unsworth[1], **Robert J. Lock**
Immunology and Immunogenetics, Pathology Sciences, Southmead Hospital, North Bristol NHS Trust, Bristol, United Kingdom
[1]Corresponding author: e-mail address: joe.unsworth@nbt.nhs.uk

Contents

Abstract

Food allergy (IgE-mediated hypersensitivity) is a common clinical problem affecting approximately 15% of children in the Western world. These hypersensitivity reactions tend to be "immediate" (typically within minutes of food exposure), and clinical features may range from mild to life threatening (anaphylaxis). Detailed clinical history is critical to correct diagnosis. Available laboratory tests have limitations not least poor positive predictive value and limited repertoire. Laboratory tests should support clinical diagnosis not vice versa.

1. FOOD ALLERGY—A GROWING PROBLEM

Fortunately, while Type 1 (IgE mediated) hypersensitivity is extremely common, with affected children in every local school, fatalities are extremely rare (approximately 1 per 5 million in the general population in the UK) [1]. Hospital admissions in the UK for suspected acute allergic events are increasing [2,3]. Severe, potentially life-threatening food allergy is more likely where a patient has concurrent poorly controlled asthma. Introduction of community Epinephrine autoinjectors is regarded as mandatory in many cases (e.g., where patients have previously had or are deemed

Advances in Clinical Chemistry, Volume 65
ISSN 0065-2423
http://dx.doi.org/10.1016/B978-0-12-800141-7.00006-1

to be at risk of anaphylaxis), but there is no evidence that fatalities from food allergy have been significantly reduced by this measure [4].

Over the last 25 years, prevalence has increased. Regarding food allergy specifically, prevalence in the USA is up to 6% in children and 4% in adults [5]. In the industrialized West, more than 1% of children have peanut allergy [6]. Reasons for this are much debated and likely multifactorial, including changes in weaning, food processing, etc. Symptoms typically occur in infancy. Fortunately, with increasing age, many of the sensitivities (egg, wheat, dairy (largely cow's milk)) are outgrown. By contrast, nut and peanut allergies tend to persist in adulthood. With increasing age, new food allergy is less likely, but exceptions include, for example, the so-called oral allergy (or "pollen–fruit") syndromes where pollen hypersensitivity leads to cross-reaction with raw uncooked fruits and vegetables carrying the same cross-reacting protein.

Ninety percent of true Type 1 (IgE) food-related hypersensitivity is accounted for by allergy to a limited range of foods including peanut, tree nuts, fish, shellfish, wheat, soya, dairy, and egg [7]. Clinically significant IgE responses are directed (generally) against protein allergens.

Food allergy presents a challenge because many patients, and some doctors, incorrectly and illogically ascribe a wide range of diverse symptoms to "food allergy." Symptoms occurring more that 1 h after food or symptoms including bloating, abdominal pain, and diarrhea are not likely to be due to true food allergy. More convincing symptoms tend to be associated with the upper gastrointestinal tract including oral irritation or itching, mouth swelling, nausea, and vomiting. Associated rapid onset body-wide urticaria/angioedema is seen in the majority but confusion arises because most urticaria/angioedema presenting in adults will be nonallergic and unrelated to food [8].

The differential diagnosis requires an ability to distinguish between true (immediate, Type 1 hypersensitive) food allergy and other problems, including food intolerance. The former requires previous exposure to allow acquisition of immunological memory, is dose independent, and will occur on every exposure even with only tiny quantities of allergic protein (allergen). By contrast, with intolerance, food-associated symptoms may be delayed, dose dependent, and variable.

Sadly, the currently available diagnostic laboratory blood tests have many limitations including restricted repertoire, low sensitivity (false-negative test; it has been reported that 10–25% of patients with negative serology may have clinical reactivity [9]), but (more problematically) false-positive tests

(low specificity). In this context, a clinically false-positive test is defined as demonstration of the presence of specific IgE to an allergen without clinical reactivity to that allergen on challenge testing (sensitization not sensitivity). In addition, true technical false-positive results are commonly encountered in patients with eczema, where high background baseline IgE level may be associated with unconvincing multiple weak false-positive results across a range of IgE-specific food tests. In contrast to the above, in this context, the limitation is with the technology to remove the nonspecific IgE from the testing system in patients with total IgE >5000 kU/L (the upper limit of the reference range for adults being in the order of 160 kU/L in most studies [10]). Patients disappointed by these limitations may be tempted to try unorthodox tests, (e.g., testing hair samples, using IgG-based tests, etc.) often generating misleading or meaningless data, supporting unlikely speculative food associations, or worse, new unproven food links. Food avoidance when excessive may cause nutritional deficiency and damage health.

The best approach is for a trained clinician to take an exact and detailed medical history and only then consider laboratory allergy tests. For example, an adverse reaction 24 h after eating two peanuts cannot on temporal grounds be "immediate hypersensitivity" (normal reaction occurs within a few minutes) and testing for IgE-specific nut or peanut allergy would be a waste of time and money, or worse may generate false-positive results (e.g., by cross-reactive carbohydrate antibodies, see Section 2.2.1.1.5). By contrast, a patient admitted to ITU with asthma and stridor, urticarial rash, and collapse a few minutes after eating shellfish requires ongoing shellfish avoidance even if initial allergy testing gives apparently reassuring negative results.

2. TESTING FOR FOOD ALLERGY

2.1. Serological testing—Standard methods

We have recently reviewed this elsewhere [10].

2.1.1 Total IgE concentration

Measurement of total serum IgE is technically easy, but of questionable value when investigating food allergy. An elevated total IgE level is neither necessary nor sufficient to support a diagnosis. Total IgE levels within the normal range does not exclude food allergy. The World Health Organization provides a reference for total serum IgE (international reference preparation 75/702) [11]. Normal ranges are age, gender, and smoking related [12–14].

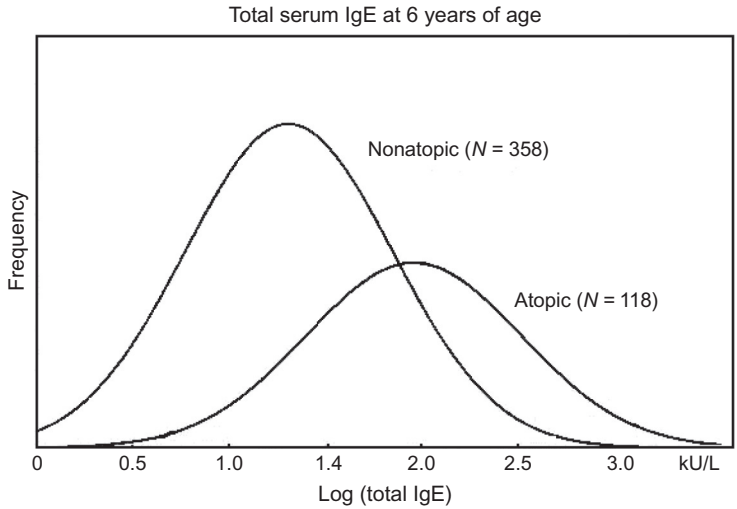

Figure 6.1 Overlap of the atopic and nonatopic populations at age 6 years. *Reproduced courtesy of the* Journal of Pediatrics *(Elsevier) from Kulig* et al. *[15].*

We use a cutoff of IgE of <113 kU/L for adults. However, the atopic (those with combinations of asthma, eczema, or hayfever) and nonatopic populations overlap and cannot be reliably distinguished on the basis of total IgE, although the former group do tend to have significantly higher levels of IgE (Fig. 6.1, reproduced by kind permission of Elsevier [15]). Total IgE has a low-positive predictive value and is a poor screen for atopy [16]. The negative predictive value of a normal IgE for atopy is unacceptably low.

2.1.2 Allergen-specific IgE antibody

These investigations remain the mainstay despite their limitations. As discussed in our recent review [10], the target allergen, extract, or allergen mix is fixed to a solid-phase carrier—paper disk (e.g., RAST, radioallergosorbent test), plastic surface (ELISA, other), cellulose (e.g., ImmunoCAP, Thermo Fisher Diagnostics), or a fluid phase polymer (e.g., Immulite, Diagnostic Products Corporation). The paper disk and ELISA offer the least binding capacity and therefore lead to more false-negative results. Single purified allergens, allergen extracts, or allergen mixes (purified, extracted, or a combination thereof) can be used. It is important to note that extracts will contain a mixture of allergens and other components, for example, carbohydrates. Patients may react to either the allergenic or the nonallergenic components, the latter will again contribute to clinical false positivity. In

addition, the more complex the mix, and hence the more allergens included, the lower the sensitivity for a given allergen (assuming equal proportions) and again risk of false-negative results. Importantly, titer does not relate to nor predict the degree of clinical sensitivity or risk. This underlines the key importance of first taking a clear clinical history to underpin the interpretation of subsequent laboratory tests. Microarrays which can test for reactivity to many (90–100) individual proteins simultaneously have recently been introduced [17,18]. The concern here is that the testing is not directed by the history; testing for specific IgE to 90–100 allergens tested increases the chance of clinical false positivity (as the assay detects sensitization not clinical reactivity to an allergen).

Specific IgE tests lack agreed international standards. Assays have used functional "standards," typically based on a single donor serum with (apparent/presumed) specificity to a specific allergen and a designated arbitrary value. More recently, assays for specific IgE calibrate to the international reference preparation 75/702 in mass equivalent terms.

Screening tests include the commonest allergen sources and proponents argue that negative values can be useful because of generally high negative predictive values. One POCT system has been approved by the US Food and Drug Administration using a panel of 10 common inhalant allergens (ImmunoCAP Rapid System, Phadia) [19]. None to our knowledge has yet been approved for food allergy, however.

In children and adolescents, 85% of all food allergies are accounted for by three allergens: cow's milk, hen's egg, and peanut [9]. Correspondence between different manufacturers' assays for specific IgE to food allergens is clearly less than ideal. Table 6.1 gives examples of the variability of sensitivity and specificity of IgE assays for some common food allergens, as documented.

Peanut is the most common reported cause of fatal or near fatal reactions to food. In children, high levels of specific IgE to peanut (>15 kU_A/L) have a high-predictive value for clinical allergy to peanut (92% positive predictive value, 95% confidence interval: 74–99%) using cautious medically supervised food challenge (at clinical risk) as gold standard [27]. Similarly, clinical reactivity to hen's egg white was predicted with >95% certainty when specific IgE was >6 kU_A/L [25]. For cow's milk allergy, Sampson and Ho [28] found that clinical reactivity was predicted with >95% certainty when specific IgE was >32 kU_A/L. However, it is important to note that not all studies agree on these limits (e.g., Celik-Bilgili *et al.* [22]), and the chosen cutoff is dependent on the population studied and the laboratory assay used, as well

Table 6.1 Examples of sensitivity and specificity of IgE assays, as reported by different centers, for some common food allergies

Source	Allergen	Sensitivity (%)	Specificity (%)	Method
Rottem *et al.* [20]	Cow's milk	67.9	70.4	Immulite, DPC, Los Angeles, CA, USA
Norgaard *et al.* [21]	Cow's milk	100	71	CAP, Pharmacia, Uppsala, Sweden
		75	71	Magic Lite, ALK-Abelló, Hørsholm, Denmark
		75	71	Matrix Food Aero, Abbott Laboratories, Abbot Park, IL, USA
Celik-Bilgili *et al.* [22]	Cow's milk	83	53	CAP, Pharmacia
Ricci *et al.* [23]	Cow's milk	91	70	ImmunoCAP, Pharmacia
		82	74	ADVIA Centaur, Bayer Corporation, New York, USA
Guilloux *et al.* [24]	Peanut	100	73	Immulite, 3gAllergy, DPC
		100	46	ImmunoCAP, Pharmacia
Norgaard *et al.* [21]	Hen's egg white	100	33	CAP, Pharmacia
		100	33	Magic Lite, ALK-Abello
		89	17	Matrix Food Aero, Abbott Laboratories
Celik-Bilgili *et al.* [22]	Hen's egg white	97	51	CAP, Pharmacia
Ricci *et al.* [23]	Hen's egg white	94	64	ImmunoCAP, Pharmacia
		88	52	ADVIA Centaur, Bayer Corporation
Celik-Bilgili *et al.* [22]	Wheat	79	38	CAP, Pharmacia
Majamaa *et al.* [25]	Wheat	20	93	CAP, Pharmacia

Table 6.1 Examples of sensitivity and specificity of IgE assays, as reported by different centers, for some common food allergies—cont'd

Source	Allergen	Sensitivity (%)	Specificity (%)	Method
Celik-Bilgili *et al.* [22]	Soy	69	50	CAP, Pharmacia
Giampietro *et al.* [26]	Soy	69	83	RAST, Pharmacia

DPC, diagnostic products corporation; RAST, radioallergosorbent test.
Sensitivity is based on a positive assay in patients who develop unequivocal features of Type 1 hypersensitivity (e.g., rash and abdominal pain) on food challenge.
Specificity is determined from those in whom there are no features of Type 1 hypersensitivity on administration of placebo.
DPC and Bayer now both Siemens Healthcare Diagnostics, Deerfield, IL, USA.
Pharmacia now part of Thermo Fisher Scientific Inc., Waltham, MA, USA.
Reproduced from Lock and Unsworth [10], courtesy of the *Annals of Clinical Biochemistry*, with kind permission of SAGE Publications Ltd.

as confounding factors such as concurrent eczema. These results have assisted clinical practice, but recently reliability has been improved by employing component-resolved diagnostics (CRD), not only for peanut but for a range of other foods too.

Another reason that researchers moved toward CRD (covered in detail in Section 2.2.1) was because of recognized cross-reacting antibodies. Some recognize similar epitopes in proteins from different species ("panallergens," e.g., birch pollen Bet v 1, and its homologues including, e.g., Mal d 1 (apple), and other PR-10 proteins found in many fruits including apple and peach). Shellfish tropomyosin is another panallergen that may provoke an allergic response to several species (e.g., shrimp, crab, crayfish) and yet may also cross react with house dust mite [29]. Clinical examples of cross reactions include the oral allergy or pollen/fruit syndrome and latex Hev b 1/fruit cross-reactivity, including kiwi and banana.

2.1.3 Skin prick testing

Skin prick testing is effectively a simple, useful, qualitative, and point of care test. In a clinic setting, skin prick testing generally gives equivalent results to parallel blood testing and can be used to corroborate or check unexpected results. However, there are limitations to the use of these tests, as outlined below:

- Skin prick testing for IgE-specific antibody is not suitable for use in all patients, for example, those taking medications including antihistamines or those with extensive eczema or dermographism.
- The repertoire of available allergen sources is more limited than blood testing.
- Generally, the assay is safe with mild local reactions being common, but patients may very rarely have severe systemic reactions.
- Concordance between skin prick and serological tests is variable, at least in part because the allergen sources differ.
- The biggest drawback of these tests is that "false positives" are common. The Isle of Wight population-based study of 981 infants showed that 1.2% (15 cases) were test positive against nuts, but only half were clinically sensitive to nuts on cautious nut challenge under medical supervision [30].

2.2. Serological testing—Advanced methods

2.2.1 Component-resolved diagnostics

The technologies using whole extracts of allergen sources have been used for many years and have become an established adjunct to investigating the allergic patient. However, they are unable to distinguish between a reaction to a nonallergenic component and an allergenic component; that is, they detect sensitization but not sensitivity. Up to 50% of patients have asymptomatic sensitization [31].

Over the last 15 years, assays have been developed to look at reactions to individual proteins so-called CRD. These may be purified natural proteins or those derived from recombinant DNA technology. Individual components are named for the Latin of the corresponding source, for example, Ara h 1 is protein 1 from peanut, *Arachis hypogaea*. Formats include individual assays for single components through to multiplex arrays on biochips. In addition, some preparations are available for skin prick testing. Some specific areas of food allergy investigation have been enhanced by the introduction of these assays and are discussed below.

2.2.1.1 Plant food proteins

Plants and their seeds contain many different proteins. These may be characterized by function, and reactivity to different groups of proteins is more or less likely to predict clinical symptoms. There are five major groups, discussed below.

2.2.1.1.1 PR-10 proteins There are 14 families of PR (pathogenesis-related) proteins, about half of which are known to contain allergens. The archetypal PR-10 protein is Bet v 1, the major allergen from *Betula verrucosa*, the white birch. PR-10 proteins have a wide distribution in the plant kingdom. They are upregulated as stress proteins, but their exact function is still uncertain [32]. Similar PR-10 proteins (Bet v 1 homologues) are found in many foods including hazelnut (*Corylus avellana*, Cor a 1), peach (*Prunus persica*, Pru p 1), celery (*Apium graveolens*, Api g 1), soy bean (*Glycine max*, Gly m 4), apple (*Malus domestica*, Mal d 1), and peanut (Ara h 8). Patients sensitized to Bet v 1 in birch pollen may also be sensitized to other PR-10 proteins (cross-reactivity). In Northern Europe, reactions to foods via birch sensitivity are generally relatively mild and life-threatening anaphylaxis is rare [33]. Typically, milder oral allergy syndrome (OAS) is seen, where the patient has local symptoms associated with the oropharynx but no systemic features. This restricted reactivity may result from the lability of the PR-10 proteins which are degraded before absorption in the gut. These proteins are readily denatured by heat so that when present in well-cooked food, they are expected to be rendered harmless.

However, there are reports to indicate that some patients may additionally have more severe reactions, notably to soy milk *Gly m4* in birch-sensitized patients [34–36].

2.2.1.1.2 Profilins These are also called *Bet v 2* homologues. Profilins are 12–15 kDa actin-binding proteins. They are one of the main causes of cross-reactivity in pollens and plant foods [37] and are considered a panallergen [38]. Although it is possible to assess reactivity to other profilins (e.g., Olive tree, *Olea europea*, Ole e 2 and Timothy grass, *Phleum pratense*, Phl p 12), this is not particularly helpful in clinical practice [39].

2.2.1.1.3 Nonspecific lipid transfer proteins (PR-14 proteins) Lipid transfer proteins (LTPs) are classified in to two subgroups by molecular weight: LTP1 (9 kDa) and LTP2 (7 kDa). Although LTPs are demonstrated to bind lipids, their *in vivo* function remains unclear [40]. It has been suggested that they may play a role in plant defense, possibly by inducing permeabilization of fungal membranes [41] or by mobilization of lipid reserves [42]. LTPs are very heat-stable, although allergenicity can be reduced by severe heat treatment [43].

Reactions to LTP appear to be more common in the Mediterranean area [44], with clinical cross-reactivity being described between foods and pollens. However, although less common in birch-endemic areas, clinical

reactivity to LTP is still important in some patients, for example, Flinterman *et al.* [45]. Clinically, there are thus similarities with the North European pollen/fruit syndrome, but this Southern Europe variant is clinically more severe, not prevented by cooking, and due to different protein cross-reactivities.

The peach protein Pru p 3 is the predominant LTP in terms of patients' specific IgE [46,47]. As with profilins and PR10 proteins, there is significant cross-reaction between species, owing to structural homology. Reactions may be systemic (anaphylaxis) or more localized (oropharynx, skin, or gastrointestinal) [48]. Of interest, a recent study by Pascal *et al.* [49] suggested that 40% of those suffering anaphylaxis required the involvement of a cofactor (most frequently nonsteroidal anti-inflammatory drugs (NSAIDs)). In some cases (11 of 34 cases), the cofactor was an absolute requirement for reactivity as well as modulating the severity of the response [49]. The mechanism by which cofactors enable clinical reactivity is unclear. It has been suggested that effects on mucosal permeability by NSAIDs or exercise, resulting in higher antigen exposure, are key [50,51].

As seen in other food allergic responses, there does not appear to be any correlation between LTP-specific IgE concentration in the serum and the severity of symptoms seen [49,52]. As with Bet v 1 and OAS, it has been suggested that the LTP-syndrome also arises as a primary response to pollen. The primary source of sensitization appears to be the plane tree (*Planatus acerifolia*). This seems to be the case both for peach sensitivity (Pru p 3) [49] and for hazelnut sensitivity (Cor a 8) [53,54]. However, it should be noted that LTPs from other pollen sources have also been implicated: olive (Ole e 7) [55], pellitory (*Parietaria judaica*, Par j 1, Par j 2) [55–57], and mugwort (*Artemisia vulgaris*, Art v 3) [56,58].

2.2.1.1.4 Storage proteins Specific IgE to storage proteins is often associated with severe reactions, including anaphylaxis. These are proteins found in seeds. They are often stable and heat resistant. They include Ara h 2 (2S albumin from peanut), Ses I 1 (7S globulin from sesame, *Sesamum indicum*), Gly m 6 (11S globulin from soy), Jug r 2 (vicilin from walnut, *Juglans regia*), and gliadins from wheat.

2.2.1.1.5 Cross-reactive carbohydrate determinants Allergic responses to elements other than the protein core of glycoproteins are well described. They are prone to significant cross-reactivity. Cross-reactive carbohydrate determinants (CCDs) are not restricted to plant proteins, and

antibodies may interfere with assays to whole extracts, for example, to bee and wasp venoms. There are two types of CCD described.

The first is galactose-alpha-1,3-galactose (α-gal). This may have clinical importance and is discussed later in Section 2.2.1.2.6.

The second CCD is of the MUX type, containing mannose, glucose, and xylose. These are often clinically irrelevant and may give clinically false-positive results when using, for example, whole peanut extracts. It is possible to check for the presence of MUX-type CCD using commercial reagents, for example, bromelain from pineapple (*Ananus comosus*, nAna c 2).

2.2.1.2 CRD in specific food allergy

The following is not a complete summary of all the assays studied in this area (well over 100 specific IgE CRD components are described). Rather, this represents the areas which have had, or may have, the greatest impact on diagnostics.

2.2.1.2.1 Peanut The peanut 2S albumin storage protein Ara h 2 has emerged as the most important allergen in peanut, with a high specificity and positive predictive value for severe reaction on oral challenge [59,60]. Indeed, it has been suggested that, on the basis of the Ara h 2 result, challenges could be reduced by over 50%. The other storage proteins Ara h 1 and Ara h 3 are also important allergens [61], but in practice do not add a great deal to the Ara h 2 result. The LTP Ara h 9 has been suggested to be important in the Mediterranean area [61–63], but a contrasting study [64] suggests the storage proteins are still the more important, as is seen elsewhere in the world.

In contrast to the above, single sensitization to the profilin Ara h 8 is indicative of no or mild symptoms on oral challenge [65].

2.2.1.2.2 Hazelnut Hazelnut allergy may vary from mild to severe. The former is largely caused by sensitization to pollen, predominantly birch, and is associated with the Bet v 1 homologue Cor a 1 or the profilin Cor a 2. More severe symptoms are associated with antibodies to Cor a 8 (LTP) and Cor a 9 (11S globulin) and possibly Cor a 11 (7S globulin) [66]. Interestingly, severity relates in part to the age of clinical presentation. Young children present predominantly with reactivity to the storage protein (Cor a 9) or the cupin (Cor a 11) and a more severe clinical outcome on ingestion of hazelnut, whereas adults presentation is most likely related to OAS due to reactivity to the Bet v 1 homologue Cor a 1.04 [67,68] and clinically milder.

2.2.1.2.3 Hen's egg Egg white contains the more important allergens: ovomucoid (Gal d 1), ovalbumin (*Gal d 2*), ovotransferrin/conalbumin (Gal d 3), and lysozyme (Gal d 4) [69]. Ovomucoid, which comprises 10–15% of the protein in egg white, is very thermostable and digestion stable, whereas the others are more labile. It has been shown that children with egg allergy high concentrations of specific IgE to Gal d 1 are predictive of those that will have a positive oral provocation challenge [70,71]. Continuing reactivity to sequential epitopes on Gal d 1 is associated with persistence of egg allergy beyond childhood [72].

2.2.1.2.4 Wheat Hypersensitivity or intolerance to wheat in manifest is several different ways, including celiac disease, and other immune or non-immune mechanisms. We will only consider IgE-mediated allergy in this chapter. Most cases will present following oral exposure, but inhalation (including Baker's asthma) and contact sensitivity are other mechanisms.

Wheat allergy may occur in infancy, as a typical type 1 reaction, with a rapid onset of symptoms. Less commonly, a cofactor, as described below, is required [73]. Specific IgE reactivity to a variety of wheat proteins is recognized including alpha-gliadins, beta-gliadins, gamma-gliadins, and omega-gliadins [74]. Reactivity to omega-5-gliadin is associated with more severe reactions including anaphylaxis.

2.2.1.2.5 Food-dependent exercise-induced anaphylaxis Food-dependent exercise-induced anaphylaxis (FDEIA) is a further example of a requirement for a cofactor to induce an allergic reaction (see Section 2.2.1.1.3). In this case, the most common presentation is a patient who can either eat a given food or take exercise without problem, unless the exercise is taken soon after (within an hour or two) eating a specific food, for example, wheat. In 90%, symptoms will be manifest within 30 min of starting exercise [75]. The first description was of shellfish-induced anaphylaxis [76]. Since then, many other foods have been implicated in many cases in the literature. Other triggers, for example temperature, have also been implicated in some cases (e.g., Refs. [77,78]). The underlying mechanisms are still unclear. The best characterized FDEIA is wheat-dependent exercise-induced anaphylaxis (WDEIA). In WDIEA, omega-5-gliadin (*Triticum aestivum*, Tri a 19) is the critical allergen and specific IgE to omega-5-gliadins is frequently found in patients' serum [73].

2.2.1.2.6 Red meat allergy Galactose-alpha-1,3-galactose (α-gal) is a sugar structure found in glycoproteins and glycolipids from all mammals

except primates, including humans. Allergy to red meat (beef, pork, lamb) usually involves delayed-type reactions (3–6 h postingestion), and this should raise suspicion of sensitization to α-gal [79–81]. The reason for the delay is unknown but may be related to the route of entry of the allergen which, it has been suggested, can be via the lymphatic system [82]. There is some evidence to suggest sensitization occurs via tick bites, with the southern and eastern US states being most affected [79,80]. However, no commercially available test exists for this antibody.

2.2.1.3 The practical application of CRD

While the use of CRD has greatly advanced our understanding of particular target allergens and mechanisms involved in type 1 hypersensitivity reactions, the day-to-day impact in the clinic has been relatively small. Many further studies are required to test, refine, and potentially extend the diagnostic worth of CRD. In the majority of cases, historical standard assays (specific IgE to unpurified allergen sources (e.g., hen's egg white and cow's milk) or SPT) are sufficient to confirm the diagnosis suspected from a good clinical history. In a few cases, CRD may obviate the need for food challenge testing. These include Ara h 2 for peanut allergy and ovomucoid (Gal d 1) for persistent hen's egg allergy. Specific IgE to omega-5-gliadin is more sensitive than whole wheat extract for the diagnosis of WDEIA. In addition, there are some examples of complex allergy where a knowledge of sensitization patterns (e.g., PR-10 vs. LTP sensitivity) might direct specific immunotherapy for example, [83]. However, we must remain alert to the inevitable consequence of more testing, that is, more clinically false-positive results and not necessarily the improved sensitivity and specificity for which we had hoped [84].

2.2.2 Mast cell tryptase

Mast cell tryptase (MCT) is a major component of mast cell granules. Normally, low levels are found in the serum. When released from mast cells, it has a short (2–2.5 h) half-life. It is present persistently in serum in high concentration when the total body mass of mast cells has increased (mastocytosis), or transiently (elevation lasting less than 24 h) when large numbers of mast cells degranulate simultaneously following a severe allergic reaction, for example, IgE-mediated anaphylaxis following intravenous drug injection in a sensitized hypersensitive (allergic) individual, or a life-threatening food allergy with systemic features. Anaphylaxis is a "severe, life-threatening generalized, or systemic hypersensitivity

reaction" [85]. Symptoms of anaphylaxis include laryngeal or pharyngeal edema, bronchospasm, and hypotension, but most episodes also include skin rashes and mucosal changes [86].

Measurement of serum histamine is unhelpful owing to the very short half-life in circulation postreaction. In contrast, serum MCT may be very useful to confirm a true anaphylactic reaction. MCT enters the blood stream postreaction and is at a peak 1–2 h postreaction, declining to normal concentration by 24 h [87]. As the initial peak may be missed, UK guidance is that samples should be taken "as soon as possible after emergency treatment has started" and "a second sample ideally within 1–2 h (but no later than 4 h) from the onset of symptoms" [86,88]. It is important to record the time of reaction and the time of sampling for correct interpretation of the data.

These recommendations are based on data from adults. Although data for children are sparse, the guidelines recommend a similar regime could be applied and clinicians should consider taking blood for MCT, in particular, where the trigger is thought to be venom-related, drug-related, or idiopathic [88]. We see no logical reason why the recommendations in adults should not also apply to children or why they should not also apply where food is the suspected trigger. If MCT is elevated at presentation, a further baseline sample for MCT should be obtained to exclude the rare diagnosis of mastocytosis.

Mastocytosis is a rare genetically determined clonal proliferation. Mutations are typically somatic, so cases can present early in childhood or in adults but without a familial pattern. Mast cell proliferation can be cutaneous only or systemic (confirmed by bone marrow examination) [89].

Where a suspected anaphylactic death has occurred, the *postmortem* MCT may be helpful to confirm. Particular care must be taken in the interpretation of *postmortem* MCT. The result may be affected by cause of death, sampling time *postmortem*, and the site from which the sample was taken. Several studies show that MCT is higher in nonanaphylaxis *postmortem* samples than in living control samples [90–93]. MCT from heart samples is more often elevated than those from femoral sites [94]. While very high levels are generally considered to be supportive of a diagnosis of anaphylaxis, they may be found outside of this context [90,95]. A recent publication advocates the use of aortic samples. An MCT >110 μg/L had a sensitivity of 80% and a specificity of 92% for anaphylaxis-associated death using this approach [93]. Whether in cases of death caused by trauma the MCT tryptase is elevated as compared to controls remains controversial. Edston and van Hage-Hamsten [90] showed

a clear association. In contrast, McLean-Tooke *et al.* [93] did not find an association of elevated MCT with trauma. Similarly, the effect of opiates (which may directly activate mast cells) on *postmortem* MCT remains unresolved. Fineschi *et al.* [96] identified elevated MCT in a number of cases of drug overdose, whereas McLean-Tooke *et al.* [93] saw no such association.

It is important to note that low or normal levels of MCT cannot exclude anaphylaxis, either *premortem* or *postmortem*. Low MCT is well described in particular patients with likely food-induced anaphylaxis [97,98]. In conclusion, moderate elevations of MCT postmortem are quite common. Elevated postmortem MCT may be supportive of a diagnosis of anaphylaxis, but the result must be interpreted in the context of the accompanying history.

2.3. Food challenge

This is regarded as the gold standard for diagnosis, and standardized protocols have been developed (e.g., Ref. [99]). Literally the proof is in the eating, but there are limitations and significant risks including risk of serious adverse allergic reaction. Hence, where food allergy based on clinical history seems likely, particularly in a high risk patient with brittle asthma, food challenge even under medical supervision is unethical and contraindicated. IgE-based food testing beforehand with negative, that is, normal, results makes an uneventful, safe challenge more likely.

Outside ethically approved research studies, this time-consuming, manpower-dependent, potentially risky approach is restricted to cases where food allergy is unlikely and where knowing the definitive answer is important, for example, in a malnourished patient on an unhealthy restricted diet. Even then, challenge needs to be medically supervised, with written consent, and with a robust way of defining a positive clinical challenge, not relying only on ill-defined symptoms. In an ideal world, because of patient anxiety or suggestibility, challenge should be placebo controlled and blinded adding to the complexity and practicality. Patients need supervision for at least 1 h postchallenge before deciding whether a patient may safely be allowed home.

2.4. Basophil activation tests

Like mast cells, basophils may be activated via IgE receptors on the cell surface leading to release of mediators including histamine, leukotriene C4, interleukin (IL)-4 and IL-13 [100,101]. Traditionally, functional assays for basophils relied on measurements of the released mediators, particularly

histamine and leukotriene and collecting cell supernatants for factor-specific quantitative assays [102,103]. These are practically problematical and have been restricted to specialist laboratories.

Recently, flow cytometric methods have instead been developed that are technically simpler and more accessible to many routine laboratories [104]. They rely on the observation that the secretory granule-associated protein CD63 (also known as 53 kDa tetraspanin or lysosomal-associated membrane glycoprotein 3 (LAMP-3)) is upregulated on basophil degranulation [105]. Generally, expression correlates well with histamine release [105,106], although not in all situations [104,107]. A rapid and transient expression of CD230c may also be seen, notably where the stimulus does not result in anaphylactic degradation (e.g., IL-3) [108].

Assays have reasonable sensitivity, with the CD63 basophil activation test in the order of 60–80% [100,109] and the leukotriene release assay in the order of 65% [109]. Although the flow-based assays have been used in some studies related to food allergy [109,110], perhaps their most extensive and promising use has been in investigating drug allergy [111,112] where existing alternative laboratory tests are very limited.

These assays require significant hands-on time and have no international standards and no external quality assessment scheme. As such they are difficult to maintain and we do not advocate their use outside specialist flow laboratories with access to allergy expertise, and certainly their role in food allergy is unproven.

2.5. Unconventional assays for allergy testing

2.5.1 IgG class antifood antibodies

There are several studies advocating the use of IgG antibodies to indicate sensitivity to specific foods and hence to direct an exclusion diet. With the exception of antigliadin antibodies in coeliac disease, these remain unproven in the diagnosis of food hypersensitivity, largely because many health individuals eating a normal diet have these IgG antibodies and are "sensitized" but not "sensitive."

Even in coeliac disease, the clinical utility of IgG antigliadin is limited. Specificity is poor, with the antibody being found in 15% of blood donors [113]. Current guidelines advise against the use of IgG-based assays to detect antibodies to native gliadin for the diagnosis of coeliac disease [114,115]. IgA antigliadin has better specificity for a diagnosis of coeliac disease but is likewise not sufficiently robust for diagnostic purposes.

Patients with irritable bowel syndrome (IBS) attribute their symptoms to certain foods. Such patients may modify their diet based on these beliefs risking inadequate nutrition [116]. True IgE-mediated food allergy is uncommon in IBS [117]. Atkinson *et al.* [118] suggested that IBS patients on a diet free of foods identified by the presence of IgG class antibodies showed an improved symptom score. However, this study has been criticized by others [119–121]. By contrast, another study suggested that symptoms improved using IgG4 antibodies to foods to select foods for inclusion in the diet [122]. There was no control group in this observational study. A more recent population-based study, after correction for subject characteristics and diet, found that there were no significant differences with regard to food- and yeast-specific IgG and IgG4 antibodies between subjects with IBS and controls [123]. Thus, the use of these IgG food antibody tests in the investigation of IBS is of uncertain significance.

A similar argument applies to the use of IgG antigliadin assays in ataxia. Studies have shown that both IgG antigliadin and IgG class antibodies to other food proteins are seen in both patient and control groups equally [124,125]. As with the disease-specific finding of antitissue transglutaminase (type 2 transglutaminase) in coeliac disease [126], there is currently much interest in antibodies to transglutaminase 6 in so-called gluten ataxia [127]. However, ataxia may not be the only setting for these antibodies [128] and further studies are required before we can come to a full understanding of the relevance of these findings.

In all, we remain unconvinced that there is sufficient evidence to support the use of specific IgG assays to foods, either for diagnosis or for the direction of elimination diets.

2.5.2 Other unconventional assays

There are a number of other tests offered for allergy testing. These include kinesiology, electrodermal testing, the leukocytotoxic test, and hair testing.

Kinesiology relies on the unfounded assumption that holding a vial of an allergen source in one hand will lead to muscle weakness in the contralateral arm. A controlled trial by Garrow [129] showed the assay to be unreliable for food allergy diagnosis. Similarly, Lüdtke *et al.* [130] showed the assay to be unreliable for venom allergy, concluding "results suggest that the use of Health Kinesiology as a diagnostic tool is not more useful than random guessing."

Electrodermal testing (Vegatesting) is based on an unsubstantiated hypothesis that in patients with allergy, there are associated changes to

electrical skin resistance. A double-blind placebo-controlled trial showed that electrodermal testing cannot distinguish between atopic and nonatopic individuals defined by skin prick tests [131]. Similarly, Semizzi *et al.* [132] determined that, in a double-blind placebo-controlled trial, electrodermal testing could not correctly detect respiratory allergy. There is no reason to believe electrodermal testing would be any more effective in the diagnosis of food allergy.

Leukocytotoxic tests involve cumbersome assays and observing changes in cell morphology on the addition of specific allergen to whole blood. There is no theoretical basis for this assay. It is subjective, nonstandardized, and nonreproducible [133–136]. In our opinion, there is no justification in its use.

Hair analysis has a limited use in identifying long-term exposure to toxic substances including heavy metals and drugs, although there are serious limitations to the analyses which make interpretation difficult. For example, it has been suggested that cadmium exposure from environmental tobacco smoke may be associated with asthmatic status [137]. Hair analysis may also help in the investigation of exposure to drugs [138]. However, there is no evidence to support its use in food allergy testing [139–141].

Hence, we support the view expressed by Niggemann and Grüber [142] that "to date, no complementary or alternative diagnostic procedure can be recommended as a meaningful element in the diagnostic work-up of allergic diseases."

3. CONCLUSIONS

IgE-based laboratory tests for food allergy remain error prone, especially with regard to clinical false-positive results and unnecessary food avoidance. Recent advances in CRD using purified and better defined allergens have provided more specific and reliable assays in some niche areas (e.g., peanut Ara h 2), and these can be of great clinical value in helping safely predict whether a food challenge can safely proceed or not. However, overall, the benefit of CRD has been limited. There remains no alternative to a carefully documented clinical history, taking care to note time intervals between food exposure, and symptom onset and type. History should dictate test selection not vice versa. Patients are often surprised by the limitations of the best available tests, and this may unfortunately tempt them to try alternative, unorthodox testing which only tends to cloud the issue. This is an

area for cautious use of existing IgE food-based assays, selective use of CRD, and more research using well-defined clinical study groups.

REFERENCES

[1] R.S. Pumphrey, R.S. Pumphrey, Fatal anaphylaxis in the UK, 1992–2001, Novartis Found. Symp. 257 (2004) 116–128.
[2] R.J. Mullins, Anaphylaxis: risk factors for recurrence, Clin. Exp. Allergy 33 (8) (2003) 1033–1040.
[3] R. Gupta, A. Sheikh, D.P. Strachan, H.R. Anderson, Time trends in allergic disorders in the UK, Thorax 62 (1) (2007) 91–96.
[4] D.J. Unsworth, Adrenaline syringes are vastly over prescribed, Arch. Dis. Child. 84 (2001) 410–411.
[5] S.H. Sicherer, H.A. Sampson, Food allergy, J. Allergy Clin. Immunol. 117 (2006) S470–S475.
[6] S.H. Sicherer, D.Y. Leung, Advances in allergic skin disease, anaphylaxis, and hypersensitivity reactions to foods, drugs, and insects in 2009, J. Allergy Clin. Immunol. 125 (2010) 85–97.
[7] H.A. Sampson, Food allergy. Part 1: immunopathogenesis and clinical disorders, J. Allergy Clin. Immunol. 103 (5 Pt 1) (1999) 717–728.
[8] F. Madsen, J. Attermann, A. Linneberg, Epidemiology of non-hereditary angioedema, Acta Derm. Venereol. 92 (2010) 475–479.
[9] H.A. Sampson, Utility of food-specific IgE concentrations in predicting symptomatic food allergy, J. Allergy Clin. Immunol. 107 (2001) 891–896.
[10] R.J. Lock, D.J. Unsworth, Food allergy—which tests are worth doing and which are not? Ann. Clin. Biochem. 48 (2011) 300–309.
[11] WHO Expert Committee on Biological Standardization, Human Serum Immunoglobulin E. 658, 21Technical Report Series, World Health Organization, Geneva, 1981.
[12] Handbook of Clinical Immunochemistry, Immunoglobulins and immunodeficiency., in: A. Milford Ward, J. Sheldon, A. Rowbottom, G.D. Wild (Eds.), Handbook of Clinical Immunochemistry, PRU Publications, Sheffield, 2007.
[13] O. Zetterstrom, S.G.O. Johansson, IgE concentrations measured by PRIST, in serum of healthy adults and in patients with respiratory allergy, Allergy 36 (1981) 537–547.
[14] A. Carosso, M. Bugiani, E. Migliore, J.M. Antò, R. DeMarco, Reference values of total serum IgE and their significance in the diagnosis of allergy in young European adults, Int. Arch. Allergy Immunol. 142 (2007) 230–238.
[15] M. Kulig, U. Tacke, J. Forster, et al., Serum IgE levels during the first 6 years of life, J. Pediatr. 134 (4) (1999) 453–458.
[16] M.R. Perkin, D.P. Strachan, W. Ho, G. Lack, ALSPAC Study Team, The predictive value of early life total immunoglobulin E measurement in identifying atopic children in a population-based birth cohort study, Paediatr. Allergy Immunol. 17 (2006) 118–124.
[17] R. Hiller, S. Laffer, C. Harwanegg, et al., Microarrayed allergen molecules: diagnostic gatekeepers for allergy treatment, FASEB J. 16 (2002) 414–416.
[18] B. Jahn-Schmid, C. Harwanegg, R. Hiller, et al., Allergen microarray: comparison of microarray using recombinant allergens with conventional diagnostic methods to detect allergen-specific serum immunoglobulin E, Clin. Exp. Allergy 33 (2003) 1443–1449.
[19] http://www.accessdata.fda.gov/cdrh_docs/pdf8/k081830.pdf (last accessed 11 February 2014).

[20] M. Rottem, D. Shostak, S. Foldi, The predictive value of specific immunoglobulin E on the outcome of milk allergy, Isr. Med. Assoc. J. 10 (2008) 862–864.
[21] A. Norgaard, C. Bindslev-Jensen, P. Stahl Skov, L.K. Poulsen, Specific serum IgE in the diagnosis of egg and milk allergy in adults, Allergy 50 (1995) 636–647.
[22] S. Celik-Bilgili, A. Mehl, A. Verstege, et al., The predictive value of specific immunoglobulin E levels in serum for the outcome of oral food challenges, Clin. Exp. Allergy 35 (2006) 268–273.
[23] R. Ricci, M. Capelli, R. Miniero, G. Menna, P. Dillon, M. Masi, A comparison of different allergometric tests, skin prick test, Pharmacia UniCAP and ADVIA Centaur, for diagnosis of allergic diseases in children, Allergy 58 (2003) 38–45.
[24] L. Guilloux, M. Morisset, F. Codreanu, L. Parisot, D.A. Moneret-Vautrin, Peanut allergy diagnosis in the context of grass pollen sensitization for 125 patients: roles of peanut and cross-reactive carbohydrate determinants specific IgE, Int. Arch. Allergy Immunol. 149 (2009) 91–97.
[25] H. Majamaa, P. Moisio, K. Holm, K. Turjanmaa, Wheat allergy: diagnostic accuracy of skin prick and patch tests and specific IgE, Allergy 54 (1999) 851–856.
[26] P.G. Giampietro, V. Ragno, S. Daniele, A. Cantani, M. Ferrara, L. Businco, Soy hypersensitivity in children with food allergy, Ann. Allergy 69 (1992) 143–146.
[27] G. Roberts, G. Lack, Diagnosing peanut allergy with skin prick testing and specific IgE testing, J. Allergy Clin. Immunol. 115 (2005) 1291–1296.
[28] H.A. Sampson, D.G. Ho, Relationship between food-specific IgE concentrations and the risk of positive food challenges in children and adolescents, J. Allergy Clin. Immunol. 100 (1997) 444–451.
[29] C. Radauer, M. Bublin, S. Wagner, A. Mari, H. Breiteneder, Allergens are distributed into few protein families and possess a restricted number of biochemical functions, J. Allergy Clin. Immunol. 121 (2008) 847–852.
[30] S.M. Tariq, M. Stevens, S.M. Matthews, S. Ridout, R. Twiselton, D.W. Hide, Cohort study of peanut and nut sensitisation by age of 4 years, BMJ 313 (1996) 514–517.
[31] J.A. Chapman, I.L. Berstein, R.E. Lee, et al., Food allergy: a practice parameter, Ann. Allergy 96 (2006) S1–S68.
[32] H. Fenandes, K. Michalska, M. Sikorski, M. Jaskolski, Structural and functional aspects of PR-10 proteins, FEBS J. 280 (2013) 1169–1199.
[33] M. Fernández-Rivas, S. Bolhaar, E. González-Mancebo, et al., Apple allergy across Europe: how allergen sensitization profiles determine the clinical expression of allergies to plant foods, J. Allergy Clin. Immunol. 118 (2006) 481–488.
[34] P. Kosma, S. Sjölander, E. Landgren, M.P. Borres, G. Hedlin, Severe reactions after the intake of soy drink in birch pollen-allergic children sensitized to Gly m 4, Acta Pedriatr. 100 (2011) 305–306.
[35] M. Berneder, M. Bublin, K. Hoffmann-Sommergruber, T. Hawranek, R. Lang, Allergen chip diagnosis for soy-allergic patients: Gly m 4 as a marker for severe food-allergic reactions to soy, Int. Arch. Allergy Immunol. 161 (2013) 229–233.
[36] L.F. De Swert, R. Gadisseur, S. Sjölander, M. Raes, J. Leus, E. Van Hoeyveld, Secondary soy allergy in children with birch pollen allergy may cause both chronic and acute symptoms, Pediatr. Allergy Immunol. 23 (2012) 117–123.
[37] R. Van Ree, V. Voitenko, W.A. van Leeuwen, R.C. Aalberse, Profilin is a cross-reacting allergen in pollen and vegetable food, Int. Arch. Allergy Immunol. 98 (1992) 97–104.
[38] R. Valenta, M. Duchene, C. Ebner, et al., Profilins constitute a novel family of functional plant pan-allergens, J. Exp. Med. 175 (1992) 377–385.
[39] D. Villalta, R. Asero, Sensitization to the pollen pan-allergen profilin. Is the detection of immunoglobulin E to multiple homologous proteins from different sources clinically useful? J. Investig. Allergol. Clin. Immunol. 20 (2010) 591–595.

[40] M. Egger, M. Hauser, A. Mari, F. Ferreira, G. Gadermaier, The role of lipid transfer proteins in allergic diseases, Curr. Allergy Asthma Rep. 10 (2010) 326–335.
[41] M.C. Regente, A.M. Giudici, J. Villalaín, L. de la Canal, The cytotoxic properties of a plant lipid transfer protein involve membrane permeabilization of target cells, Lett. Appl. Microbiol. 40 (2005) 183–189.
[42] A.G. Gonorazky, M.C. Regente, L. de la Canal, Stress induction and antimicrobial properties of a lipid transfer protein in germinating sunflower seeds, J. Plant Physiol. 162 (2005) 618–624.
[43] A.I. Sancho, N.M. Rigby, L. Zuidmeer, et al., The effect of thermal processing on the IgE reactivity of the non-specific lipid transfer protein from apple, Mal d 3, Allergy 60 (2005) 1262–1268.
[44] G. Salcedo, R. Sánchez-Monge, A. Diaz-Perales, Plant non-specific lipid transfer proteins as food and pollen allergens, Clin. Exp. Allergy 34 (2004) 1336–1341.
[45] A.E. Flinterman, J.H. Akkerdaas, G.F. den Hartog Jager, et al., Lipid transfer protein-linked hazelnut allergy in children from a non-Mediterranean birch-endemic area, J. Allergy Clin. Immunol. 121 (2) (2008) 423–428.
[46] R. Rodrigues-Alves, A. Lopez, M.C. Pereira-Santos, et al., Clinical, anamnestic and serological features of peach allergy in Portugal, Int. Arch. Allergy Immunol. 149 (2009) 65–73.
[47] R. Asero, R.L. Antonicelli, A. Arena, et al., EpidemAAITO: features of food allergy in Italian adults attending allergy clinics: a multi-centre study, Clin. Exp. Allergy 39 (2009) 547–555.
[48] E.A. Pastorello, A.M. Robino, Clinical role of lipid transfer proteins in food allergy, Mol. Nutr. Food Res. 48 (2004) 356–362.
[49] M. Pascal, R. Muñoz-Cano, Z. Reina, et al., Lipid transfer protein syndrome: clinical pattern, co-factor effect and profile of molecular sensitization to plant-foods and pollens, Clin. Exp. Allergy 42 (2012) 1529–1539.
[50] G.P. Lambert, M. Boylan, J.P. Laventure, A. Bull, S. Lanspa, Effect of aspirin and ibuprofen on GI permeability during exercise, Int. J. Sports Med. 28 (2007) 72.
[51] H. Matsuo, S. Kaneko, Y. Tsujino, et al., Effects of non-steroidal anti-inflammatory drugs (NSAIDs) on serum allergen levels after wheat ingestion, J. Dermatol. Sci. 53 (2009) 241–243.
[52] R. Asero, A. Arena, L. Cecchi, et al., Are IgE levels to foods other than rosaceae predictive of allergy in lipid transfer protein-hypersensitive patients? Int. Arch. Allergy Immunol. 155 (2011) 149–154.
[53] E. Enrique, A. Cisteró-Bahíma, B. Bartolomé, et al., Platanus acerifolia pollinosis and food allergy, Allergy 57 (2002) 351–356.
[54] F. Schocker, D. Lüttkopf, S. Scheurer, et al., Recombinant lipid transfer protein Cor a 8 from hazelnut: a new tool for in vitro diagnosis of potentially severe hazelnut allergy, J. Allergy Clin. Immunol. 113 (2004) 141–147.
[55] D. Barber, F. de la Torre, F. Feo, et al., Understanding patient sensitization profiles in complex pollen areas: a molecular epidemiological study, Allergy 63 (2008) 1550–1558.
[56] M.A. Ciardiello, P. Palazzo, M.L. Bernardi, et al., Biochemical, immunological and clinical characterization of a cross-reactive nonspecific lipid transfer protein 1 from mulberry, Allergy 65 (2010) 597–605.
[57] E. Scala, C. Alessandri, M.L. Bernardi, et al., Cross-sectional survey on immunoglobulin E reactivity in 23,077 subjects using an allergenic molecule-based microarray detection system, Clin. Exp. Allergy 40 (2010) 911–921.
[58] M. Lombardero, F.J. García-Sellés, F. Polo, et al., Prevalence of sensitization to Artemisia allergens Art v 1, Art v 3 and Art v 60 kDa. Cross-reactivity among Art v 3 and other relevant lipid-transfer protein allergens, Clin. Exp. Allergy 34 (2004) 1415–1421.

[59] N. Nicolaou, C. Murray, D. Belgrave, M. Poorafshar, A. Simpson, A. Custovic, Quantification of specific IgE to whole peanut extract and peanut components in prediction of peanut allergy, J. Allergy Clin. Immunol. 127 (2011) 684–685.
[60] E. Eller, C. Bindslev-Jensen, Clinical value of component-resolved diagnostics in peanut-allergic patients, Allergy 68 (2013) 190–194.
[61] A. Vereda, M. van Hage, S. Ahlstedt, et al., Peanut allergy: clinical and immunologic differences among patients from 3 different geographic regions, J. Allergy Clin. Immunol. 127 (2011) 603–607.
[62] S. Krause, G. Reese, S. Randow, et al., Lipid transfer protein (Ara h 9) as a new peanut allergen relevant for a Mediterranean allergic population, J. Allergy Clin. Immunol. 124 (2009) 771–778.
[63] G. Javaloyes, M.J. Goikoetxea, I. García Núñez, et al., Performance of different in vitro techniques in the molecular diagnosis of peanut allergy, J. Investig. Allergol. Clin. Immunol. 22 (2012) 508–513.
[64] M. Pedrosa, T. Boyano-Martínez, M.C. García-Ara, T. Caballero, S. Quirce, Peanut seed storage proteins are responsible for clinical reactivity in Spanish peanut-allergic children, Pediatr. Allergy Immunol. 23 (2012) 654–659.
[65] A. Asarnoj, C. Nilsson, J. Lidholm, et al., Peanut component Ara h 8 sensitization and tolerance to peanut, J. Allergy Clin. Immunol. 130 (2012) 468–472.
[66] A.E. Flinterman, J.H. Akkerdaas, A.C. Knulst, R. van Ree, S.G. Pasmans, Hazelnut allergy: from pollen-associated mild allergy to severe anaphylactic reactions, Curr. Opin. Allergy Clin. Immunol. 8 (2008) 261–265.
[67] K.J. De Knop, M.M. Verweij, M. Grimmelikhuijsen, et al., Age-related sensitization profiles for hazelnut (Corylus avellana) in a birch-endemic region, Pediatr. Allergy Immunol. 22 (1 Pt 2) (2011) e139–e149.
[68] D.G. Ebo, M.M. Verweij, V. Sabato, M.M. Hagendorens, C.H. Bridts, L.S. De Clerck, Hazelnut allergy: a multi-faced condition with demographic and geographic characteristics, Acta Clin. Belg. 67 (2012) 317–321.
[69] H. Everberg, P. Brostedt, H. Oman, S. Bohman, R. Movérare, Affinity purification of egg-white allergens for improved component-resolved diagnostics, Int. Arch. Allergy Immunol. 154 (2011) 33–41.
[70] T. Boyano-Martínez, C. García-Ara, J.M. Díaz-Pena, M. Martín-Esteban, Prediction of tolerance on the basis of quantification of egg white-specific IgE antibodies in children with egg allergy, J. Allergy Clin. Immunol. 110 (2002) 304–309.
[71] D.E. Marriage, M. Erlewyn-Lajeunesse, D.J. Unsworth, A.J. Henderson, Unscrambling egg allergy: the diagnostic value of specific IgE concentrations and skin prick tests for ovomucoid and egg white in the management of children with Hen's egg allergy. ISRN Allergy (2012) 1–6, http://dx.doi.org/10.5402/2012/627545, Article ID 627545.
[72] K.M. Järvinen, K. Beyer, L. Vila, L. Bardina, M. Mishoe, H.A. Sampson, Specificity of IgE antibodies to sequential epitopes of hen's egg ovomucoid as a marker for persistence of egg allergy, Allergy 62 (2007) 758–765.
[73] E. Morita, H. Matsuo, Y. Chinuki, et al., Food-dependent exercise-induced anaphylaxis—importance of omega-5 gliadin and HMW-glutenin as causative antigens for wheat-dependent exercise-induced anaphylaxis, Allergol. Int. 58 (2009) 493–498.
[74] A.S. Tatham, P.R. Shewry, Allergens in wheat and related cereals, Clin. Exp. Allergy 38 (2008) 1712–1726.
[75] N.A. Shadick, M.H. Liang, A.J. Partridge, et al., The natural history of exercise-induced anaphylaxis: survey results from a 10-year follow-up study, J. Allergy Clin. Immunol. 104 (1999) 123–127.

[76] R.M. Maulitz, D.S. Pratt, A.L. Schocket, Exercise induced anaphylactic reaction to shellfish, J. Allergy Clin. Immunol. 63 (1979) 433–434.
[77] T. Shimizu, H. Furumoto, E. Kinoshita, et al., Food-dependent exercise-induced anaphylaxis occurring only in winter, Dermatology 200 (2000) 279.
[78] E.J. Jo, M.S. Yang, Y.J. Kim, et al., Food-dependent exercise-induced anaphylaxis occurred only in a warm but not in a cold environment, Asia Pac. Allergy 2 (2012) 161–164.
[79] S.P. Commins, H.R. James, L.A. Kelly, et al., The relevance of tick bites to the production of IgE antibodies to the mammalian oligosaccharide galactose-α-1,3-galactose, J. Allergy Clin. Immunol. 127 (2011) 1286–1293.
[80] J.L. Kennedy, A.P. Stallings, T.A. Platts-Mills, et al., Galactose-α-1,3-galactose and delayed anaphylaxis, angioedema, and urticaria in children, Pediatrics 131 (2013) e1545–e1552.
[81] M. Morisset, C. Richard, C. Astier, et al., Anaphylaxis to pork kidney is related to IgE antibodies specific for galactose-alpha-1,3-galactose, Allergy 67 (2012) 699–704.
[82] H. Saleh, S. Embry, A. Nauli, S. Atyia, G. Krishnaswamy, Anaphylactic reactions to oligosaccharides in red meat: a syndrome in evolution, Clin. Mol. Allergy 10 (1) (2012) 5.
[83] R.E. Rossi, G. Melioli, G. Monasterolo, et al., Sensitization profiles in polysensitized patients from a restricted geographical area: further lessons from multiplexed component resolved diagnosis, Eur. Ann. Allergy Clin. Immunol. 43 (2011) 171–175.
[84] O.D. Wolthers, M. Staberg, A comparison of the usefulness of the multiple allergen simultaneous test-chemiluminescent assay as compared to the phadia immunocap IgE test panel system in children and adolescents, Recent Pat. Inflamm. Allergy Drug Discov. 7 (2013) 96–99.
[85] S.G.O. Johansson, T. Bieber, R. Dahl, et al., A revised nomenclature for allergy for global use: report of the nomenclature review committee of world allergy organization, J. Allergy Clin. Immunol. 3 (2004) 832–836.
[86] Working Group of the Resuscitation Council (UK), Emergency Treatment of Anaphylactic Reactions. Guidelines for Healthcare Providers, Resuscitation Council (UK), London, 2008.
[87] L.B. Schwartz, Diagnostic value of tryptase in anaphylaxis and mastocytosis, Immunol. Allergy Clin. North Am. 26 (2006) 451–463.
[88] NICE clinical guideline 134, Anaphylaxis: Assessment to Confirm an Anaphylactic Episode and the Decision to Refer After Emergency Treatment for a Suspected Anaphylactic Episode, National Institute for Health and Clinical Excellence, London, UK, 2011.
[89] S.J. Fuller, New insights into the pathogenesis, diagnosis, and management of mastocytosis, Haematol. Oncol. Clin. North Am. 26 (2012) 1143–1168.
[90] E. Edston, M. van Hage-Hamsten, Mast cell tryptase and hemolysis after trauma, Forensic Sci. Int. 131 (2003) 8–13.
[91] E. Edston, O. Eriksson, M. van Hage, Mast cell tryptase in postmortem serum-reference values and confounders, Int. J. Legal Med. 121 (2007) 275–280.
[92] D.E. Mayer, A. Krauskopf, W. Hemmer, K. Moritz, R. Jarisch, C. Reiter, Usefulness of post mortem determination of serum tryptase, histamine and diamine oxidase in the diagnosis of fatal anaphylaxis, Forensic Sci. Int. 212 (2011) 96–101.
[93] A. McLean-Tooke, M. Goulding, C. Bundell, J. White, P. Hollingsworth, Postmortem serum tryptase levels in anaphylactic and non-anaphylactic deaths, J. Clin. Pathol. 67 (2) (2014) 134–138.
[94] E. Edston, M. van Hage-Hamsten, beta-Tryptase measurements post-mortem in anaphylactic deaths and in controls, Forensic Sci. Int. 93 (1998) 135–142.
[95] B. Randall, J. Butts, J.F. Halsey, Elevated postmortem tryptase in the absence of anaphylaxis, J. Forensic Sci. 40 (1995) 208–211.

[96] V. Fineschi, R. Cecchi, F. Centini, L.P. Reatelli, E. Turillazzi, Immunohistochemical quantification of pulmonary mast-cells and post-mortem blood dosages of tryptase and eosinophil cationic protein in 48 heroin-related deaths, Forensic Sci. Int. 120 (2001) 189–194.
[97] H.A. Sampson, L. Mendelson, J.P. Rosen, Fatal and near-fatal anaphylactic reactions to food in children and adolescents, N. Engl. J. Med. 327 (1992) 380–384.
[98] R.Y. Lin, L.B. Schwartz, A. Curry, et al., Histamine and tryptase levels in patients with acute allergic reactions: an emergency department-based study, J. Allergy Clin. Immunol. 106 (1 Pt 1) (2000) 65–71.
[99] C. Bindslev-Jensen, B.K. Ballmer-Weber, U. Bengtsson, et al., Standardization of food challenges in patients with immediate reactions to foods—position paper from the European Academy of Allergology and Clinical Immunology, Allergy 59 (2004) 690–697.
[100] D.A. Moneret-Vautrin, J. Sainte-Laudy, G. Kanny, S. Fremont, Human basophil activation measured by CD63 expression and LTC4 release in IgE-mediated food allergy, Ann. Allergy Asthma Immunol. 82 (1999) 33–40.
[101] J. Kleine-Tebbe, S. Erdmann, E.F. Knol, D.W. MacGlashan Jr., L.K. Poulsen, B.F. Gibbs, Diagnostic tests based on human basophils: potentials, pitfalls and perspectives, Int. Arch. Allergy Immunol. 141 (2006) 79–90.
[102] A.D. Crockard, M. Ennis, Basophil histamine release tests in the diagnosis of allergy and asthma, Clin. Exp. Allergy 31 (2001) 345–350.
[103] P. Demoly, B. Lebel, B. Arnoux, Allergen-induced mediator release tests, Allergy 58 (2003) 553–558.
[104] A.L. de Weck, M.L. Sanz, P.M. Gamboa, Diagnostic tests based on human basophils: more potentials and perspectives than pitfalls, Int. Arch. Allergy Immunol. 146 (2008) 177–189.
[105] E.F. Knol, F.P. Mul, H. Jansen, J. Calafat, D. Roos, Monitoring human basophil activation via CD63 monoclonal antibody 435, J. Allergy Clin. Immunol. 88 (3 Pt 1) (1991) 328–338.
[106] M.J. Metzelaar, P.L. Wijngaard, P.J. Peters, J.J. Sixma, H.K. Nieuwenhuis, H.C. Clevers, CD63 antigen. A novel lysosomal membrane glycoprotein, cloned by a screening procedure for intracellular antigens in eukaryotic cells, J. Biol. Chem. 266 (1991) 3239–3245.
[107] D. MacGlashan Jr., Expression of CD203c and CD63 in human basophils: relationship to differential regulation of piecemeal and anaphylactic degranulation processes, Clin. Exp. Allergy 40 (2010) 1365–1377.
[108] F. Hennersdorf, S. Florian, A. Jakob, et al., Identification of CD13, CD107a, and CD164 as novel basophil-activation markers and dissection of two response patterns in time kinetics of IgE-dependent upregulation, Cell Res. 15 (2005) 325–335.
[109] S.M. Erdmann, N. Heussen, S. Moll-Slodowy, H.F. Merk, B. Sachs, CD63 expression on basophils as a tool for the diagnosis of pollen-associated food allergy: sensitivity and specificity, Clin. Exp. Allergy 33 (2003) 607–614.
[110] D.G. Ebo, O. Ahrazem, G. Lopez-Torrejon, C.H. Bridts, G. Salcedo, W.J. Stevens, Anaphylaxis from mandarin (Citrus reticulata): identification of potential responsible allergens, Int. Arch. Allergy Immunol. 144 (2007) 39–43.
[111] D.G. Ebo, S. Goossens, F. Opsomer, C.H. Bridts, W.J. Stevens, Flow assisted diagnosis of anaphylaxis to hyaluronidase, Allergy 60 (2005) 1333–1334.
[112] D.G. Ebo, R.D. Wets, T.K. Spiessens, C.H. Bridts, W.J. Stevens, Flow assisted diagnosis of anaphylaxis to patent blue, Allergy 60 (2005) 703–704.
[113] C.D. Pengiran Tengah, R.J. Lock, D.J. Unsworth, A.J. Wills, Multiple sclerosis and occult gluten sensitivity, Neurology 62 (2004) 2326–2327.

[114] National Institute for Health and Clinical Excellence, Coeliac Disease: Recognition and Assessment of Coeliac Disease. National Institute for Health and Clinical Excellence, London, 2009.www.nice.org.uk/CG86.
[115] S. Husby, S. Koletzko, I.R. Korponay-Szabó, for the ESPGHAN Working Group on Coeliac Disease Diagnosis, on behalf of the ESPGHAN Gastroenterology Committee, et al., European Society for Pediatric Gastroenterology, Hepatology, and Nutrition Guidelines for the Diagnosis of Coeliac Disease, J. Pediatr. Gastroenterol. Nutr. 54 (2012) 136–160.
[116] K.W. Monsbakken, P.O. Vandvik, P.G. Farup, Perceived food intolerance in subjects with irritable bowel syndrome—etiology, prevalence and consequences, Eur. J. Clin. Nutr. 60 (2006) 667–672.
[117] A. Morcos, T. Dinan, E.M. Quigley, Irritable bowel syndrome: role of food in pathogenesis and management, J. Dig. Dis. 10 (2009) 237–246.
[118] W. Atkinson, T.A. Sheldon, N. Shaath, P.J. Whorwell, Food elimination based on IgG antibodies in irritable bowel syndrome: a randomized controlled trial, Gut 53 (2004) 1459–1464.
[119] J.O. Hunter, Food elimination in IBS: the case for IgG testing remains doubtful, Gut 54 (2005) 1203.
[120] J.E. Mawdsley, P. Irving, R. Makins, IgG antibodies to foods in IBS, Gut 54 (2005) 567.
[121] W.A. Sewell, IgG food antibodies should be studied in similarly treated groups, Gut 54 (2005) 566.
[122] S. Zar, L. Mincher, M.J. Benson, D. Kumar, Food-specific IgG4 antibody-guided exclusion diet improves symptoms and rectal compliance in irritable bowel syndrome, Scand. J. Gastroenterol. 40 (2005) 800–807.
[123] S.C. Ligaarden, S. Lydersen, P.G. Farup, IgG and IgG4 antibodies in subjects with irritable bowel syndrome: a case control study in the general population, BMC Gastroenterol. 12 (2012) 166.
[124] M. Abele, L. Schöls, S. Schwartz, T. Klockgether, Prevalence of antigliadin antibodies in ataxia patients, Neurology 60 (2003) 1674–1675.
[125] R.J. Lock, D.S.N.A. Pengiran Tengah, D.J. Unsworth, J.J. Ward, A.J. Wills, Ataxia, peripheral neuropathy, and anti-gliadin antibody. Guilt by association? J. Neurol. Neurosurg. Psychiatry 76 (2005) 1601–1603.
[126] W. Dieterich, T. Ehnis, M. Bauer, et al., Identification of tissue transglutaminase as the autoantigen of celiac disease, Nat. Med. 3 (1997) 797–801.
[127] M. Hadjivassiliou, P. Aeschlimann, D.S. Sanders, et al., Transglutaminase 6 antibodies in the diagnosis of gluten ataxia, Neurology 80 (19) (2013) 1740–1745.
[128] N.G. Cascella, D. Santora, P. Gregory, D.L. Kelly, A. Fasano, W.W. Eaton, Increased prevalence of transglutaminase 6 antibodies in sera from schizophrenia patients, Schizophr. Bull. 39 (4) (2013) 867–871.
[129] J.S. Garrow, Kinesiology and food allergy, Br. Med. J. (Clin. Res. Ed.) 296 (6636) (1988) 1573–1574.
[130] R. Lüdtke, B. Kunz, N. Seeber, J. Ring, Test-retest-reliability and validity of the Kinesiology muscle test, Complement. Ther. Med. 9 (2001) 141–145.
[131] G.T. Lewith, J.N. Kenyon, J. Broomfield, P. Prescott, J. Goddard, S.T. Holgate, Is electrodermal testing as effective as skin prick tests for diagnosing allergies? A double blind, randomised block design study, BMJ 322 (2001) 131–134.
[132] M. Semizzi, G. Senna, M. Crivellaro, et al., A double-blind, placebo-controlled study on the diagnostic accuracy of an electrodermal test in allergic subjects, Clin. Exp. Allergy 32 (2002) 928–932.
[133] P. Lieberman, L. Crawford, J. Bjelland, B. Connell, M. Rice, Controlled study of the cytotoxic food test, JAMA 231 (1975) 728–730.

[134] T.E. Benson, J.A. Arkins, Cytotoxic testing for food allergy: evaluation of reproducibility and correlation, J. Allergy Clin. Immunol. 58 (1976) 471–476.
[135] C.W. Lehman, The leukocytic food allergy test: a study of its reliability and reproducibility. Effect of diet and sublingual food drops on this test, Ann. Allergy 45 (1980) 150–158.
[136] P.P. VanArsdel Jr., E.B. Larson, Diagnostic tests for patients with suspected allergic disease. Utility and limitations, Ann. Intern. Med. 110 (1989) 304–312.
[137] C.H. Razi, K.O. Akin, K. Harmanci, et al., Relationship between hair cadmium levels, indoor ETS exposure and wheezing frequency in children, Allergol. Immunopathol. (Madr.) 40 (1) (2012) 51–59.
[138] R.L. DuPont, W.A. Baumgartner, Drug testing by urine and hair analysis: complementary features and scientific issues, Forensic Sci. Int. 70 (1995) 63–76.
[139] S. Barrett, Commercial hair analysis. Science or scam? JAMA 254 (1985) 1041–1045.
[140] T.J. Sethi, M.H. Lessof, D.M. Kemeny, E. Lambourn, S. Tobin, A. Bradley, How reliable are commercial allergy tests? Lancet 1 (8524) (1987) 92–94.
[141] A.B. Kay, M.H. Lessof, Allergy: conventional and alternative concepts. A report of the Royal College of Physicians Committee on clinical immunology and allergy, Clin. Exp. Allergy 22 (Suppl. 3) (1992) 1–44.
[142] B. Niggemann, C. Grüber, Unproven diagnostic procedures in IgE-mediated allergic diseases, Allergy 59 (2004) 806–808.

CHAPTER SEVEN

Matrix Metalloproteinases in Biologic Samples

Gregory T. Jones[1]
Department of Surgery, Dunedin School of Medicine, University of Otago, Dunedin, New Zealand
[1]Corresponding author: e-mail address: greg.jones@otago.ac.nz

Contents

Abstract

Matrix metalloproteinases (MMPs) are an important class of endopeptidases, having a role in a diverse range of physiological and pathological processes. This chapter provides an overview of the key regulatory processes in MMP production and activation. The common techniques used to assess MMP activity are discussed and their various strengths and weaknesses presented. This comparison of methodologies is specifically intended to aid any investigator who wishes to determine the most appropriate analytical method for their future studies because any investigation of MMPs in biological samples should be cognizant of the key mechanisms influencing the expression and activity of these proteinases. The endogenous, preanalytic and analytic chemistry of MMP activation influences the interpretation of the various techniques widely employed throughout the literature. Therefore, the ability to accurately evaluate the true endogenous activity of MMPs is heavily dependent on a clear understanding of these processes.

Advances in Clinical Chemistry, Volume 65
ISSN 0065-2423
http://dx.doi.org/10.1016/B978-0-12-800141-7.00007-3

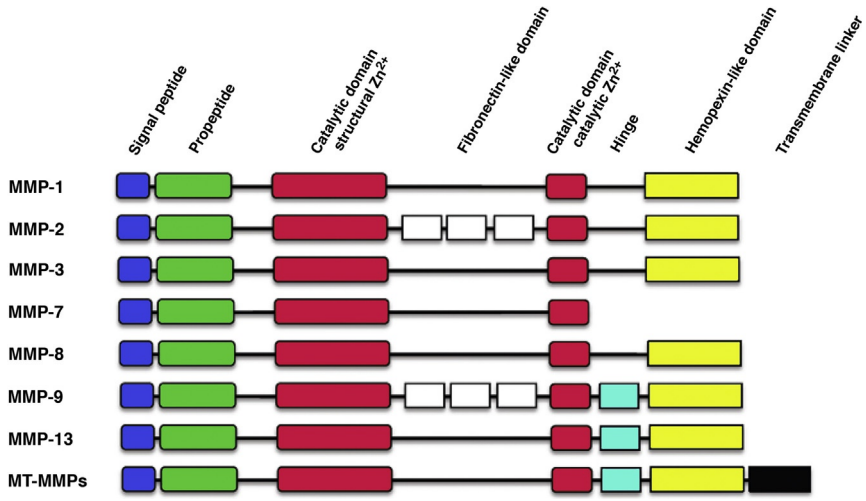

Figure 7.1 Domain structure of matrix metalloproteinases. *Adapted from Massova* et al. *[4] © Federation of American Societies for Experimental Biology (FASEB), used by permission.*

Matrix metalloproteinases (MMPs) are a subfamily of the metzincin superfamily of endogenous proteinases [1–3]. MMPs typically consist of a propeptide domain, a catalytic metalloproteinase domain, a linker (hinge region) peptide, and a hemopexin domain [1]. Common structural features consist of a zinc-binding motif (HEXXHXXGXXH) within the catalytic domain and a cysteine switch motif (PRCGXPD) within the propeptide domain [4] (Fig. 7.1). The binding of these domains to the catalytic zinc ion keeps the proenzyme in an inactive state by preventing water molecule binding, required for catalysis, to the zinc ion [1]. Understanding the nature of these activation states is key to interpreting any methodological technique that attempts to measure MMP activity.

The various members of the MMP family are subdivided based on their substrate specificities, but share common features such as having Zn^{2+} in their active catalytic site, requiring Ca^{2+} to function, and being biologically active at neutral pH [1]. Most are secreted in an inactive proform (so-called zymogen) and are inhibited by a family of endogenous inhibitors, the tissue inhibitor of matrix metalloproteinases (TIMPs). The TIMPs are a family of endogenous MMP inhibitors that appear to regulate MMP activity by binding noncovalently to the target, inhibiting functioning of the active site by interacting with the Zn^{2+} [5].

MMPs are highly conserved across animal species, and it is likely that a single primordial MMP gene was duplicated, with the resulting divergence

leading to the observed multiplicity of structure and function between different members of the MMP family [6]. While their main role appears to be proteolysis of extracellular matrix components [3], it is important to note that these proteases also have substrate specificity for cytokines, nuclear proteins, and membrane proteins. As such, MMPs play a significant role in physiological processes including procytokine and growth factor activation, DNA repair and cell death, ectodomain shedding, as well as cell migration, differentiation, and survival [2].

The key MMP subgroups, based on substrate preference and domain organization, involved in human disease are the collagenases (MMP-1, -8, -13), gelatinases (MMP-2, -9), stromelysins (MMP-3, -10), matrilysins (MMP-7, -11, -26), macrophage elastase (MMP-12), and membrane-type MMPs (MMP-14, -15, -16, -24 and GPI-anchored MMP-17, -25). There is a multiplicity of MMP substrate specificities, with the overlapping functions most-likely acting as a safeguard against any loss of regulatory control. While undoubtedly biologically advantageous to the organism, this overlap in MMP-associated proteolytic potential is a likely confounder to any MMP activity analysis [7] and should be well understood by any investigator undertaking research in this area.

1. REGULATION OF MMPs

In any experiment analyzing MMPs within biological specimens, it is important to understand the key mechanisms influencing the expression and activity of these proteinases. Because of their potentially potent physiological and pathogenic functions, MMPs are tightly regulated at the transcriptional, posttranscriptional, and posttranslational levels. In addition, protein activity is influenced by activators, inhibitors, and specific tissue-binding characteristics.

1.1. Transcriptional control

The MMP genes are under transcriptional control, with their promoters containing several *cis*-elements which can be regulated by *trans*-activators such as AP-1, SP-1, C/EBP-β, TIE, and NF-κB [8]. A number of MMPs are therefore secreted in response to growth factor mediators of remodeling, vascular stress, injury, and inflammation [9]. In contrast, MMP-2 appears to be unique in that it is not induced by inflammatory cytokines, and consequently has a more stable expression *in vivo* [10]. A schema clustering different MMP genes based on the presence or absence of TATA box and AP-1 binding sites has been suggested [8]. Since inflammatory cytokines such as IL-1 and TNFα interact with TATA box and AP-1 elements, it can be

correctly predicted that MMP genes with these elements (e.g., MMP-1, -3, -9) will be altered by inflammation [11,12].

Genetic variations within MMP promoters have been associated with altered gene expression. Generally, these variants involve alteration of *cis*-elements. For example, the −1562T polymorphism within the promoter of MMP9 alters a nuclear protein-binding complex and is associated with a 1.5-fold increase in MMP-9 expression [13]. Similarly, the −1306T variant within the promoter of MMP2 disrupts a Sp1-binding site. Consequently, the −1306C variant has a 1.4- to 2-fold increase in gene expression compared with the T variant [14]. By examining such variants, investigators have been able to implicate MMPs in various pathological conditions [13,15–17].

1.2. Posttranslational

MMPs are synthesized as preproenzymes, with the signal peptide being removed during translation. The key aspect of MMP activation requires release of the cysteine switch. A cysteine residue within the prodomain complexes with the zinc ion within the catalytic region, thereby preventing enzyme activity. The amino acid sequences surrounding both the prodomain cysteine residue and the zinc-binding ligands are highly conserved in all MMPs [18]. Removal or modification of the prosequence releases the cysteine switch, freeing the active site and allowing it to interact with water and catalytic substrate [19] (Fig. 7.2).

Depending on the specific MMP, activation may occur within the intracellular or extracellular domains or at the cellular surface. Extracellular activation typically involves a stepwise activation [21], involving an initial cleavage of a protease susceptible "bait" region, then complete removal of the remaining propeptide, often by other MMPs. Those MMPs that undergo intracellular activation utilize a furin-like proprotein convertase recognition sequence at the propeptide terminus [22]. MMPs that have undergone intracellular activation can then be secreted from the cell, or bound to the cell surface, in an active state. In such cases, the tissue activity of these MMPs is therefore dependent on tissue specific expression and/or the presence of endogenous inhibitors.

MMPs can be stored within intracellular exocytic vesicles, including those associated with the microtubular network. Disruption of the microtubules in melanoma cells results in decreased MMP secretion. This suggests that the active propulsion of MMP-containing vesicles along the microtubular network may be a key feature of the rapid, directional secretion of

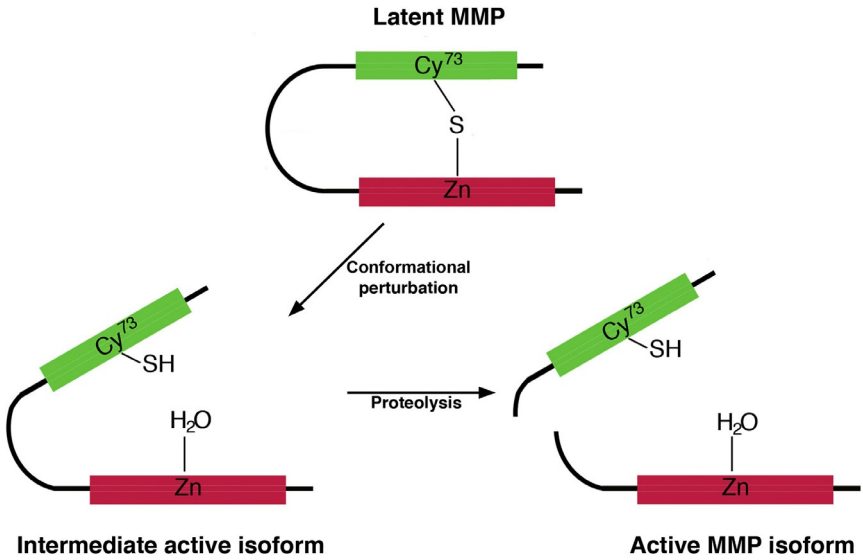

Figure 7.2 MMP activation states. Partial denaturing of the latent zymogen results in a conformational perturbation which opens the cysteine switch and forms the intermediate active isoform. Because this isoform is still attached to the propeptide, it has a higher molecular weight than the active isoform but is partially catalytically active. Consequently, both the intermediate and active isoforms are able to digest substrate in zymography gels [20]. *Adapted from Snoek-van Beurden and Von den Hoff [20] © 2009 BioTechniques, used by permission.*

MMPs during processes such as cell migration, cancer cell invasion, and three-dimensional tissue organization [23]. While MMPs clearly have a significant role in extracellular proteolytic processes, reports indicating nuclear localization of MMPs in a wide range of cell types suggest that some of these enzymes may participate in a much wider range of biological processes [24].

A wide range of biological molecules have been shown to play a role in MMP activation. These include oxidative compounds [19] and a range of proteolytic enzyme such as the serine proteinase chymase [25], urokinase plasminogen activator [26], and kallikrein [27]. Activation via other MMPs also occurs [28]; for example, MMP-2 can be activated by the membrane-type MMPs [27] and MMP-9 by MMP-3 [29,30]. Self-convertase activity has also been demonstrated, with membrane-type MMP-1 being capable of cleaving of its own prodomain at the furin cleavage motif [31].

MMPs can undergo chemical modification resulting in enzyme activation. This is an important feature that has been successfully utilized in several of the analytical techniques that will be discussed below. These chemical

activators fall into four main groups: (1) proteases (e.g., trysin and MMP autolysis), (2) conformational perturbants (e.g., detergents such as sodium dodecyl sulfate (SDS)), (3) reversible sulfhydryl-group modifiers (e.g., organomercurials such as *p*-aminophenylmercuric acetate (APMA) [32]), and (4) irreversible sulfhydryl-group modifiers (e.g., oxidants, alkylating agents) [18].

An endogenous example of such modification includes the ability of neutrophils to utilize chlorinated oxidants, such as HOCl, to activate latent MMP [33]. This reaction ability to release the cysteine switch and free the catalytic domain is a process that appears important during inflammatory-mediated MMP activation. Another important example, at least within vascular and cancer biology, is the activation cascade of urokinase, leading to conversion of plasminogen to plasmin. Plasmin appears to be an important component in prodomain cleavage and the subsequent activation of some MMPs [34,35], with inhibition of this pathway in murine genetic knockouts of urokinase [26] and plasminogen [36] resulting in altered MMP activity.

It is important to note that the MMPs can exist in an intermediate active form (Fig. 7.2), whereby the catalytic zinc ion is exposed but the prodomain remains attached to the catalytic domain. In such cases, though the MMP is potentially catalytically active, it is important to consider whether this activity is physiological or an artifact of chemical perturbation, for example, during sample analytical processing.

1.3. MMP inhibitors—The TIMPs

MMP activity is also regulated by the TIMPs, a family of two-domain proteins, which act as endogenous MMP inhibitors [37]. All mammalian TIMPs have an N-terminal domain of approximately 125 amino acids in length and a shorter (~65 amino acids) C-terminal domain. These domains are stabilized by three disulphide bonds. Though they have varied specificities, the four human TIMPs can all be considered to be broad-spectrum inhibitors of the MMPs found in humans. The four human TIMPs share around 40% sequence homology, while the most similar pair, TIMP-2 and -4, has 50% homology. While TIMP-1 has the most restricted inhibitory range, having relatively low affinity for the membrane-type MMPs, it has a higher affinity for MMP-3 and -7 than TIMP-2 and -3. TIMP-3 inhibits the broadest range of MMPs, as well as several members of the disintegrin–metalloproteinases (ADAMs and ADAMTSs). TIMP-1, -2, and -4 have

much more limited interactions with the disintegrin–metalloproteinases [37]. The biological significance of these varied affinities is demonstrated by the absence of significant abnormalities in *TIMP1* or *TIMP2* knockout mice, while mice without *TIMP3* develop significantly impaired liver [38] and lung function and have a shorter life-span [39].

The key inhibitory interaction involves a ridge insert, formed by disulfide linkage, of five residues in the TIMP N-terminal domain that fits above the MMP zinc ion catalytic domain. This occupies approximately 75% of the protein–protein interaction and displaces the water molecule needed for peptide bond hydrolysis. Although the TIMP C-terminal domains do contact with the MMP structure, in most cases, these appear to have limited roles in enzyme inhibition since truncated N-terminal TIMP fragments appear to act as high-affinity MMP inhibitors. There are exceptions, however, that indicate that the C-terminal domain also acts to increase binding affinity [40]. For example, the TIMP-2 C-terminal domain forms stabilizing interactions with the hemopexin domain of MMP-2, resulting in greater binding affinity by the full-length TIMP compared with the isolated N-TIMP domain [41]. This noninhibitory MMP–TIMP complex has been shown to have a role in cell surface activation via TIMP mediated tethering to membrane-type MMPs [42].

Several other proteins also inhibit specific members of the MMP family, including a β-amyloid precursor protein [43] and a procollagen C-proteinase enhancer protein [44] which both inhibit MMP-2. RECK, a membrane-anchored glycoprotein, negatively regulates MMP-2, -9, and membrane-type MMP-1 [45].

2. DETECTION OF MMPs IN BIOLOGICAL SAMPLES, TOTAL ZYMOGEN VERSUS ENZYMATIC ACTIVITY

When considering the assessment of MMPs within a biological sample, it is important to consider which aspect of MMP expression (total zymogen, prodomain cleaved, TIMP-bound, or endogenously active) is most biologically relevant to the research question being investigated. This is an important determinant, as direct measurement of MMP protein will not distinguish active from inactive enzyme. At this point, it is probably worth clarifying some of the terminology used regarding the various isoforms of MMPs. The term "total MMP" should be used when discussing the combined protein levels of all MMP isoforms. "Pro-MMP" levels refer to the amount of protein that has an intact propeptide domain. "Latent MMP"

refers to the summation of pro-MMP and propeptide-cleaved MMP that is also complexed with a TIMP. The term "active MMP" should be reserved for propeptide-cleaved MMP that was not endogenously bound to a TIMP. Active MMP would therefore have been enzymatically active within the tissue from which it was extracted. As described below, different analytical techniques vary in their ability to make these important distinctions.

2.1. Immunohistochemistry

The localization of MMPs and TIMPs within biological tissues is a straightforward and robust technique, particularly given the abundance of high-quality commercially available antibodies. Figures 7.3 and 7.4 show chromogenic-substrate visualized MMP-9 and -2 staining in human vascular tissues. While this technique is an excellent method to identify regions and cell types with increased MMP protein expression, it cannot typically discriminate whether the enzymes are latent or active.

2.2. Substrate zymography

Substrate zymography is a widely used technique that identifies MMPs by the degradation of their specific substrates [20]. In its most basic form samples, suitably homogenized (in the presence of a protease inhibitor such as pepstatin) and extracted, are loaded into a well on a polyacrylamide gel and the proteins separated by electrophoresis. The polyacrylamide gel is preprepared by copolymerizing it with the MMP substrate of choice

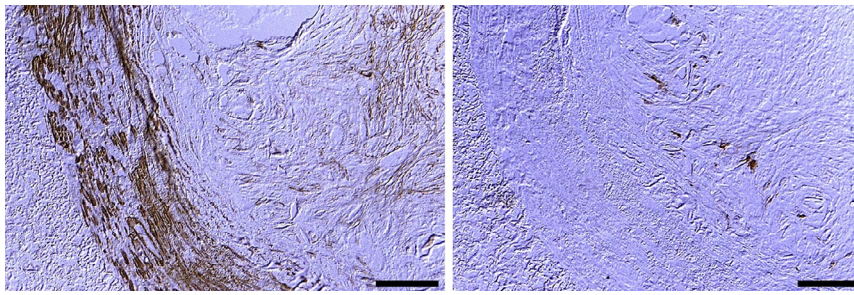

Figure 7.3 Serial sections showing smooth muscle alpha actin (left) and MMP-9 (right) immunostaining (DAB, dark reaction product) in human atherosclerotic (posterior tibial) artery. Macrophages within the atherosclertic intimal layer are MMP-9 positive. The monoclonal primary antibodies used were anti-alpha smooth muscle actin, Sigma #A2547, and anti-human MMP-9, R&D Systems, # MAB911. The surrounding tissue is not counterstained but rather tissue contrast is provided by differential interference contrast (DIC). Scale bar equals 100 μm.

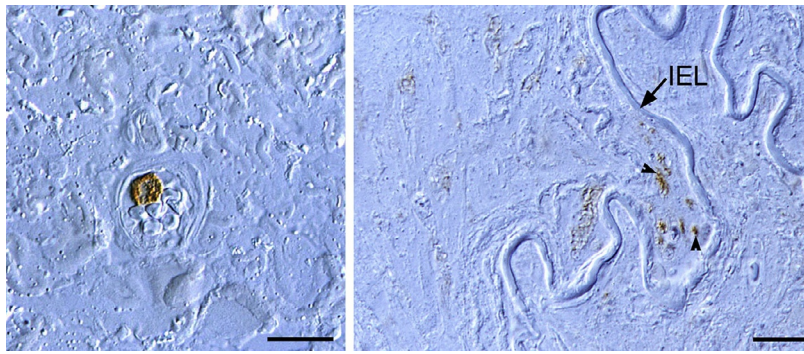

Figure 7.4 MMP-9 (left) and MMP-2 (right), immunohistochemical staining (DAB, dark reaction product) in human vascular (posterior tibial artery) tissue. The cytoplasm of a circulating monocyte within a small blood vessel is strongly MMP-9 immunopositive. MMP-2 positive staining with smooth muscle cells (arrowheads) adjacent to the internal elastic lamina (IEL) of a muscular artery. The monoclonal primary antibodies used were anti-human MMP-9 and anti-human MMP-2, R&D Systems, # MAB911 and #MAB902, respectively. Surrounding tissue contrast is provided by DIC. Scale bar equals 20 μm.

(e.g., gelatin). Electrophoresis is conducted under nonreducing conditions using SDS as the denaturing agent. Denaturing inactivates the MMPs during gel migration, after which the SDS is exchanged by washing in Triton X-100 resulting in partial renaturing and restoration of activity. As described above, SDS is able to cause conformational perturbation of the cysteine switch, a change that persists after the partial renaturation of the protein. Consequently, the zymogen is left in the intermediate active form (Fig. 7.2). The gel is then incubated at 37 °C to allow MMP-mediated substrate degradation. The gel is then stained with a protein stain (typically Coomassie blue) and the MMPs detected as unstained regions on the positively stained protein substrate background (Fig. 7.5). To confirm that the observed proteolytic activity is MMP specific, it is prudent to routinely incubate a duplicate gel in a buffer that also contains ethylenediaminetetraacetic acid (EDTA), a strong metal ion chelator and therefore a broad-spectrum MMP inhibitor. In zymography, SDS treatment plays three critical roles. First, it causes a reversible inhibition of MMP activity during electrophoresis. Second, when the MMP is partially renatured, the cysteine switch ends up in an open position so that otherwise inactive zymogen is (partially) catalytically active. Finally, it dissociates TIMPs from the catalytic domain so that MMP that was endogenously bound to a TIMP can also be catalytically active. The widely reported advantage of this technique is that it can be used to assess both pro- and "active" MMP isoforms

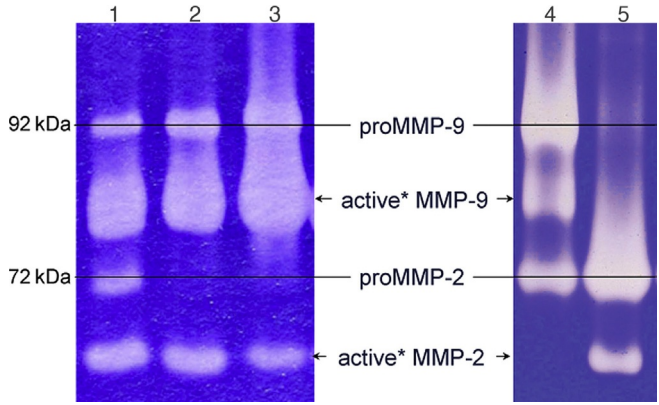

Figure 7.5 Gelatin substrate zymography. Note the absence of significant pro-MMP-2 isoforms in lanes 2 and 3 and the active isoform band in lane 4. *Because TIMPs are chemically dissociated from MMPs during the processing for this technique, it is not possible to know how much of the active isoform band would have been endogenously active (i.e., some of the MMP protein within this band is likely to have been latent *in vivo*).

within a single sample. This is because the molecular weight of the propeptide containing zymogen will be predictably higher than the "active" isoform. Although a very widely used technique, the chemical modifications to both the MMPs and MMP–TIMP complex means that quantification of zymography results should be viewed with significant caution. First, the renaturation of the protein following removal of SDS only partially restores catalytic activity of both isoforms. This may result in a failure to detect all the MMP within a sample, particularly when they are of low abundance. While gelatin substrate zymography has been shown to be remarkably sensitive for MMP-2 and -9 [46], this is not necessarily the case for other MMPs which have lower specificity for this substrate [20].

Second, it is not possible to know how much of the MMP in the "active" isoform band was endogenously bound to a TIMP. For this reason, the true quantification of latent MMP protein levels consists of the entire pro-MMP band and *a fraction* of the protein within the so-called active band. Finally, the proteolytic properties of the propeptide containing MMP isoforms, in the intermediate active state, are reduced compared to that of the active isoform. Densitometry-based quantification of the pro- and active lysis-bands should not necessarily be interpreted as direct comparison of the number of MMP molecules present.

These points notwithstanding, it is certainly possible to identify differences in isoform abundance using this method. For example, it is possible

to gain an appreciation of the relative presence or absence of a particular isoform within a given sample. As shown in Fig. 7.5, the sample run in lane 4 has no detectable lysis-band corresponding to the smaller (prodomain cleaved, so-called active) MMP-2 isoform. It is therefore possible to conclude that this sample had no (detectable) endogenous MMP-2 activity. In contrast, the lane 2 and to a lesser extent lane 3 samples appear to have undergone near complete prodomain cleavage. However, it is not possible to determine how much of the smaller isoform was truly endogenously active, since the inhibitory effects of TIMP binding cannot be accounted for in the analysis.

A range of methodological variations of the conventional gel zymography technique have now been developed. These include transfer blot, mixed substrate, two-dimensional and antibody zymography. The relative advantages and disadvantages of these technique variants are well described in a recent review by Vandooren and colleagues [47]. However, since aspects such as electrophoresis remain as integral methodological components, the issues of technique-induced sample artifacts (discussed above) largely also remain valid.

In summary, zymography is certainly a useful technique for the analysis of MMPs, albeit one that requires a careful understanding of its limitations and how best to interpret the analytical results.

2.3. *In situ* zymography

In situ zymography combines fresh-frozen histological tissue sections with substrate digestion zymography. Sites of proteolytic activity can thus be localized to specific tissue regions or cell types. Visualization may be via simple degradation of a slide coating substrate such as gelatin [48,49] or may involve cleavage of a dye-quenched substrate producing fluorescence at the site of substrate degradation [50,51]. Because this technique does not involve the same degree of chemical modification associated with gel electrophoresis, the resulting proteolytic activity is a truer reflection of the endogenous state. Care must still be taken to include the appropriate control slides, such as separate MMP and other specific protease inhibitors, to ensure that the resulting enzymatic activity is specifically MMP related. This technique is best suited to histological localization of substrate-specific MMP activity, but does not differentiate between the various MMPs capable of cleaving the substrate. When combined with immunohistochemistry, *in situ* zymography is an effective means of studying protease activity within tissue samples [47].

2.4. Western blot

Using the abundance of highly specific antibodies, that are commercially available, it is possible to accurately quantify protein levels in biological samples using Western blot analysis. A good example of how this technique can be utilized was reported by Kohrmann and colleagues [52], who examined the expression of 19 MMPs in breast cancer cells. The antibodies employed in this study recognized both the zymogen and the prodomain cleaved MMP isoforms. Because these isoforms have different molecular weights, the authors were able to report the relative abundance of both isoforms. While the relative quantification of the two related MMP protein bands does not suffer some of the same methodological issues associated with quantification of zymography bands (specifically possible differential rates of substrate digestion), there are still issues which require consideration when interpreting Western blot results. Any MMP–TIMP complexes will still be dissociated during electrophoresis; therefore, the small molecular weight band may not accurately represent endogenous activity. Separate Western blot quantification of the TIMP family members may help address this issue but only indirectly.

2.5. Conventional enzyme-linked immunosorbent assays

As with Western blot techniques, the availability of highly specific MMP and TIMP protein antibodies has resulted in a wide range of sensitive enzyme-linked immunosorbent assays (ELISA) being available to the research community. By carefully selecting antibodies that are specific to the various MMP domains or whose binding is not influenced by the MMP–TIMP complex, it is possible to accurately determine MMP protein levels. These may be measures of pro-MMP or, by selecting an antibody which binds both pro- and propeptide-cleaved MMPs, total MMP. By measuring total TIMP-1 protein in the same way, it is possible to calculate the of MMP/TIMP ratio. Such ratios have been widely used by researchers as a surrogate for MMP activity [53–55]; however, recent studies have reported a poor correlation between the MMP/TIMP ratio and MMP activity [56,57]. While this lack of coherence may be in part context dependent, for example, being more pronounced in states of inflammation [56], it nevertheless suggests that researchers should be extremely cautious about interpreting the MMP/TIMP ratio as a reliable surrogate for MMP activity.

2.6. ELISA-based MMP activity assays

As discussed above, there are a range of possible issues with zymography, Western blot, or MMP/TIMP ratio-based assessments of MMP activity. An alternative technique is to utilize immunocapture followed by a dye-quenched fluorescence substrate degradation assessment of MMP activity. Such ELISA-based activity assays have several advantages. First, MMPs are selectively and sensitively isolated, using a MMP-specific capture antibody, in such a way that there is minimal chemical modification of captured protein. Second, the capture process does not dissociate the MMP/TIMP complex so that endogenous inhibition of MMPs is maintained. Finally, the multiwell format of these assays allows large numbers of samples (including sample duplicates) to be analyzed simultaneously along with standards consisting of known concentrations of recombinant MMP. This results in a sensitive, highly quantifiable, assay for the assessment of endogenous MMP activity. The addition of a sulfhydryl-group modifier, such as APMA, is used to activate the recombinant standards but can also be added to the sample wells to determine the samples total catalytic potential. It should be noted, however, that when this is done, the resulting total (activity) MMP concentration measurement can appear higher than the promeasurement by conventional ELISA [58]. A possible explanation for this relates to the different capture antibodies used, with at least one commercial supplier stating that their selected capture antibody has a lower cross-reactivity with MMPs that are bound to a TIMP [59,60]. Regardless of the reasons, even though most of the various conventional ELISA and ELISA-based activity assays are intrinsically reliable [58], with coefficients of variance generally well below 10% [61], their respective measures of MMP levels are unlikely to be directly comparable.

It stands to reason that the most biologically important isoform is that which is endogenously active. Such propeptide-cleaved and TIMP-free isoforms are arguably best measured by ELISA-based MMP activity assays. Studies comparing both conventional ELISA measures of pro-MMP and ELISA-based activity assays have shown that endogenously active MMP levels can be altered without significant changes in the levels of the associated pro-MMP isoforms [61–63]. Such observations must be of concern when considering the numerous negative studies that have only measured pro- or total MMP protein levels. It is highly plausible that enhanced activation of MMPs without significant changes in total MMP production could be a factor in MMP-mediated biological processes.

3. CONFOUNDERS OF MMP MEASUREMENTS

A number of variables have been implicated as confounders of circulating MMP levels, including age [64], gender, white cell count [65], and certain medications [66–68]. Altered MMP expression has also been implicated in a wide range of pathophysiological conditions including obesity [69], cancer [70,71], liver fibrosis [72], rheumatoid arthritis [73], heart disease [65,74–76], and peripheral vascular disease [77].

Controversy exists regarding several preanalytical aspects of MMP assessment; in particular, the suitability of serum versus plasma [78,79], the anticoagulant used for blood plasma collection [78,80], and length of time frozen samples are stored before being analyzed [81,82].

The generation of fibrin, via intrinsic and extrinsic coagulation pathways, dose-dependently binds MMP proforms (such as MMP-1, -8, and -9). The associated plasmin activity results in an elevation of active MMP isoforms within the serum. In addition, thrombin-mediated platelet aggregation and activation also results in the release of MMPs [79]. Finally, the silica-based compounds used as coagulation accelerators in blood serum collection tubes also induce cellular release of MMPs [83]. It may therefore seem appropriate to avoid MMP analysis in serum in preference to plasma; however, this sample type is not without issues that need to be carefully considered. As MMPs are zinc-dependent enzymes, it seems reasonable to expect that metal ion chelators, such as EDTA, could influence the activity of plasma collected using such agents as anticoagulants. Indeed, lower MMP-2 activity has been reported in EDTA plasma compared with heparin plasma or serum [80]. Citrate has been shown to be associated with lower MMP-9 [84], while heparin has been reported to increase MMP-9 and TIMP-2 [85]. Probably the most informative study compared serum with different concentrations of EDTA, citrate, and heparin in plasma [86]. Although this study was limited by the use of gelatin substrate zymography (as discussed above), it nevertheless showed a clear effect, with increasing concentrations of citrate having an inverse relationship with the densitometry measurements of the propeptide-cleaved (active) isoform bands. Perhaps surprisingly, EDTA did not appear to significantly reduce MMP levels. The authors found that despite these differences, MMP measurements in all three anticoagulants correlated well with each other (Pearson's correlation coefficient $r > 0.85$) and concluded that all three anticoagulants could potentially be used to assess MMPs in blood. Nevertheless,

serum, particularly that produced with the aid of coagulation accelerators, should be avoided if possible, as this sample type appears to have the greatest variation due to release from platelets and leucocytes [78,80].

Rouy *et al.* have previously described a rapid decline in both MMP-9 protein and enzymatic activity in plasma samples stored at −80 °C for as little as 3 months, with a more gradual decline extending beyond 2 years [81]. They did not observe the same decline in TIMP-1 or MMP-2 protein levels. These observations are of significant concern to any investigator wishing to assess these analytes. This is a particularly difficult problem for the clinical researcher, for whom sample collection can occur over an extended period of time. In such studies, sample analysis is typically performed in a single batch at the completion of participant recruitment [61,87]. A recent study examining the effect of frozen storage time on MMP-9 activity was performed using the same commercial assay as the Rouy study. Although samples were not examined in the first year of storage, there was no apparent decline in samples stored from between 1 and 3 years [82]. This observation, in a large set of samples, is in striking contradiction with that of Rouy *et al.* and the results are difficult to reconcile. One explanation for the different observations is that the two studies examined plasma collected with different anticoagulants. In the Rouy *et al.* study, citrate was used, while Tarr *et al.* used heparin. As noted above, choice of anticoagulant is a potential confounder of MMP measurements, particularly pro-MMP-9 activity when assessed by substrate zymography.

Several other investigators have also examined the effect of storage temperature and repeated freeze thaws on detection of MMPs in blood samples. Kisand *et al.* [88] examined the rate of degradation of MMP-7 and TIMP-1 when stored at different temperatures and found that MMP-7 was highly stable, particularly when stored at −75 °C. Souza-Tarla *et al.* [89] examined the effect of freeze-thaw cycles and storage temperature on the stability of pro-MMP-2 and -9, and the zymographic activity of MMP-9, finding that there was no significant difference between MMPs stored at −20 or −80 °C for 1 month. Based on these observations, it would appear prudent to acknowledge the potential confounding effects of sample storage time in any study that examines MMPs. Although the inclusion of a quality control analysis examining storage time as a confounding factor should always be considered, the balance of evidence indicates that at least some MMP-related analytes can be successfully measured in appropriately stored long-term biobanked samples.

In conclusion, any investigation of MMPs in biological samples should be cognizant of the key mechanisms influencing the expression and activity of these proteinases. The chemistry of MMP activation also influences the interpretation of the various analytical techniques widely employed throughout the literature. The ability to accurately evaluate the true endogenous activity of MMPs is dependent on a clear understanding of the chemical modifications associated with all steps in the analytical process.

REFERENCES

[1] H. Nagase, R. Visse, G. Murphy, Structure and function of matrix metalloproteinases and TIMPs, Cardiovasc. Res. 69 (3) (2006) 562–573.
[2] S. Rivera, M. Khrestchatisky, L. Kaczmarek, G.A. Rosenberg, D.M. Jaworski, Metzincin proteases and their inhibitors: foes or friends in nervous system physiology? J. Neurosci. 30 (46) (2010) 15337–15357.
[3] F.X. Gomis-Ruth, Structural aspects of the metzincin clan of metalloendopeptidases, Mol. Biotechnol. 24 (2) (2003) 157–202.
[4] I. Massova, L.P. Kotra, R. Fridman, S. Mobashery, Matrix metalloproteinases: structures, evolution, and diversification, FASEB J. 12 (12) (1998) 1075–1095.
[5] K. Brew, D. Dinakarpandian, H. Nagase, Tissue inhibitors of metalloproteinases: evolution, structure and function, Biochim. Biophys. Acta 1477 (1–2) (2000) 267–283.
[6] M. Fanjul-Fernandez, A.R. Folgueras, S. Cabrera, C. Lopez-Otin, Matrix metalloproteinases: evolution, gene regulation and functional analysis in mouse models, Biochim. Biophys. Acta 1803 (1) (2010) 3–19.
[7] M.D. Sternlicht, Z. Werb, How matrix metalloproteinases regulate cell behavior, Annu. Rev. Cell Dev. Biol. 17 (2001) 463–516.
[8] C. Yan, D.D. Boyd, Regulation of matrix metalloproteinase gene expression, J. Cell. Physiol. 211 (1) (2007) 19–26.
[9] Z.S. Galis, J.J. Khatri, Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly, Circ. Res. 90 (3) (2002) 251–262.
[10] U. Ikeda, K. Shimada, Matrix metalloproteinases and coronary artery diseases, Clin. Cardiol. 26 (2) (2003) 55–59.
[11] N.R. Keller, E. Sierra-Rivera, E. Eisenberg, K.G. Osteen, Progesterone exposure prevents matrix metalloproteinase-3 (MMP-3) stimulation by interleukin-1alpha in human endometrial stromal cells, J. Clin. Endocrinol. Metab. 85 (4) (2000) 1611–1619.
[12] Y. Zhang, K. McCluskey, K. Fujii, L.M. Wahl, Differential regulation of monocyte matrix metalloproteinase and TIMP-1 production by TNF-alpha, granulocyte-macrophage CSF, and IL-1 beta through prostaglandin-dependent and -independent mechanisms, J. Immunol. 161 (6) (1998) 3071–3076.
[13] B. Zhang, S. Ye, S.M. Herrmann, P. Eriksson, M. de Maat, A. Evans, D. Arveiler, G. Luc, F. Cambien, A. Hamsten, H. Watkins, A.M. Henney, Functional polymorphism in the regulatory region of gelatinase B gene in relation to severity of coronary atherosclerosis, Circulation 99 (14) (1999) 1788–1794.
[14] S.J. Price, D.R. Greaves, H. Watkins, Identification of novel, functional genetic variants in the human matrix metalloproteinase-2 gene: role of Sp1 in allele-specific transcriptional regulation, J. Biol. Chem. 276 (10) (2001) 7549–7558.
[15] G.T. Jones, V.L. Phillips, E.L. Harris, J.I. Rossaak, A.M. van Rij, Functional matrix metalloproteinase-9 polymorphism (C-1562T) associated with abdominal aortic aneurysm, J. Vasc. Surg. 38 (6) (2003) 1363–1367.

[16] R. Okada, S. Kawai, M. Naito, A. Hishida, N. Hamajima, K. Shinchi, T. Chowdhury Turin, S. Suzuki, E.M. Mantjoro, K. Toyomura, K. Arisawa, N. Kuriyama, S. Hosono, H. Mikami, M. Kubo, H. Tanaka, K. Wakai, Japan Multi-Institutional Collaborative Cohort Study Group, Matrix metalloproteinase-9 gene polymorphisms and chronic kidney disease, Am. J. Nephrol. 36 (5) (2012) 444–450.
[17] C. Zhang, C. Li, M. Zhu, Q. Zhang, Z. Xie, G. Niu, X. Song, L. Jin, G. Li, H. Zheng, Meta-analysis of MMP2, MMP3, and MMP9 promoter polymorphisms and head and neck cancer risk, PLoS One 8 (4) (2013) e62023.
[18] H.E. Van Wart, H. Birkedal-Hansen, The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family, Proc. Natl. Acad. Sci. U. S. A. 87 (14) (1990) 5578–5582.
[19] E.B. Springman, E.L. Angleton, H. Birkedal-Hansen, H.E. Van Wart, Multiple modes of activation of latent human fibroblast collagenase: evidence for the role of a Cys73 active-site zinc complex in latency and a "cysteine switch" mechanism for activation, Proc. Natl. Acad. Sci. U. S. A. 87 (1) (1990) 364–368.
[20] P.A. Snoek-van Beurden, J.W. Von den Hoff, Zymographic techniques for the analysis of matrix metalloproteinases and their inhibitors, Biotechniques 38 (1) (2005) 73–83.
[21] H. Nagase, K. Suzuki, J.J. Enghild, G. Salvesen, Stepwise activation mechanisms of the precursors of matrix metalloproteinases 1 (tissue collagenase) and 3 (stromelysin), Biomed. Biochim. Acta 50 (4–6) (1991) 749–754.
[22] P. Stawowy, H. Meyborg, D. Stibenz, N. Borges Pereira Stawowy, M. Roser, U. Thanabalasingam, J.P. Veinot, M. Chretien, N.G. Seidah, E. Fleck, K. Graf, Furin-like proprotein convertases are central regulators of the membrane type matrix metalloproteinase-pro-matrix metalloproteinase-2 proteolytic cascade in atherosclerosis, Circulation 111 (21) (2005) 2820–2827.
[23] E.M. Schnaeker, R. Ossig, T. Ludwig, R. Dreier, H. Oberleithner, M. Wilhelmi, S.W. Schneider, Microtubule-dependent matrix metalloproteinase-2/matrix metalloproteinase-9 exocytosis: prerequisite in human melanoma cell invasion, Cancer Res. 64 (24) (2004) 8924–8931.
[24] F. Mannello, V. Medda, Nuclear localization of matrix metalloproteinases, Prog. Histochem. Cytochem. 47 (1) (2012) 27–58.
[25] K. Kishi, M. Muramatsu, D. Jin, K. Furubayashi, S. Takai, H. Tamai, M. Miyazaki, The effects of chymase on matrix metalloproteinase-2 activation in neointimal hyperplasia after balloon injury in dogs, Hypertens. Res. 30 (1) (2007) 77–83.
[26] P. Carmeliet, L. Moons, R. Lijnen, M. Baes, V. Lemaitre, P. Tipping, A. Drew, Y. Eeckhout, S. Shapiro, F. Lupu, D. Collen, Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation, Nat. Genet. 17 (4) (1997) 439–444.
[27] S. Chakraborti, M. Mandal, S. Das, A. Mandal, T. Chakraborti, Regulation of matrix metalloproteinases: an overview, Mol. Cell. Biochem. 253 (1–2) (2003) 269–285.
[28] A.Y. Strongin, I. Collier, G. Bannikov, B.L. Marmer, G.A. Grant, G.I. Goldberg, Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloprotease, J. Biol. Chem. 270 (10) (1995) 5331–5338.
[29] M.W. Olson, M.M. Bernardo, M. Pietila, D.C. Gervasi, M. Toth, L.P. Kotra, I. Massova, S. Mobashery, R. Fridman, Characterization of the monomeric and dimeric forms of latent and active matrix metalloproteinase-9. Differential rates for activation by stromelysin 1, J. Biol. Chem. 275 (4) (2000) 2661–2668.
[30] L. Sobrin, Z. Liu, D.C. Monroy, A. Solomon, M.G. Selzer, B.L. Lokeshwar, S.C. Pflugfelder, Regulation of MMP-9 activity in human tear fluid and corneal epithelial culture supernatant, Invest. Ophthalmol. Vis. Sci. 41 (7) (2000) 1703–1709.

[31] D.V. Rozanov, A.Y. Strongin, Membrane type-1 matrix metalloproteinase functions as a proprotein self-convertase. Expression of the latent zymogen in Pichia pastoris, autolytic activation, and the peptide sequence of the cleavage forms, J. Biol. Chem. 278 (10) (2003) 8257–8260.
[32] G. Galazka, L.J. Windsor, H. Birkedal-Hansen, J.A. Engler, APMA (4-aminophenylmercuric acetate) activation of stromelysin-1 involves protein interactions in addition to those with cysteine-75 in the propeptide, Biochemistry 35 (34) (1996) 11221–11227.
[33] G.J. Peppin, S.J. Weiss, Activation of the endogenous metalloproteinase, gelatinase, by triggered human neutrophils, Proc. Natl. Acad. Sci. U. S. A. 83 (12) (1986) 4322–4326.
[34] E. Hahn-Dantona, N. Ramos-DeSimone, J. Sipley, H. Nagase, D.L. French, J.P. Quigley, Activation of proMMP-9 by a plasmin/MMP-3 cascade in a tumor cell model. Regulation by tissue inhibitors of metalloproteinases, Ann. N. Y. Acad. Sci. 878 (1999) 372–387.
[35] E.N. Baramova, K. Bajou, A. Remacle, C. L'Hoir, H.W. Krell, U.H. Weidle, A. Noel, J.M. Foidart, Involvement of PA/plasmin system in the processing of pro-MMP-9 and in the second step of pro-MMP-2 activation, FEBS Lett. 405 (2) (1997) 157–162.
[36] H.R. Lijnen, B. Van Hoef, F. Lupu, L. Moons, P. Carmeliet, D. Collen, Function of the plasminogen/plasmin and matrix metalloproteinase systems after vascular injury in mice with targeted inactivation of fibrinolytic system genes, Arterioscler. Thromb. Vasc. Biol. 18 (7) (1998) 1035–1045.
[37] K. Brew, H. Nagase, The tissue inhibitors of metalloproteinases (TIMPs): an ancient family with structural and functional diversity, Biochim. Biophys. Acta 1803 (1) (2010) 55–71.
[38] F.F. Mohammed, D.S. Smookler, S.E. Taylor, B. Fingleton, Z. Kassiri, O.H. Sanchez, J.L. English, L.M. Matrisian, B. Au, W.C. Yeh, R. Khokha, Abnormal TNF activity in Timp3$^{-/-}$ mice leads to chronic hepatic inflammation and failure of liver regeneration, Nat. Genet. 36 (9) (2004) 969–977.
[39] K.J. Leco, P. Waterhouse, O.H. Sanchez, K.L. Gowing, A.R. Poole, A. Wakeham, T.W. Mak, R. Khokha, Spontaneous air space enlargement in the lungs of mice lacking tissue inhibitor of metalloproteinases-3 (TIMP-3), J. Clin. Invest. 108 (6) (2001) 817–829.
[40] M.W. Olson, D.C. Gervasi, S. Mobashery, R. Fridman, Kinetic analysis of the binding of human matrix metalloproteinase-2 and -9 to tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2, J. Biol. Chem. 272 (47) (1997) 29975–29983.
[41] J.R. Worley, P.B. Thompkins, M.H. Lee, M. Hutton, P. Soloway, D.R. Edwards, G. Murphy, V. Knauper, Sequence motifs of tissue inhibitor of metalloproteinases 2 (TIMP-2) determining progelatinase A (proMMP-2) binding and activation by membrane-type metalloproteinase 1 (MT1-MMP), Biochem. J. 372 (Pt 3) (2003) 799–809.
[42] E. Morgunova, A. Tuuttila, U. Bergmann, K. Tryggvason, Structural insight into the complex formation of latent matrix metalloproteinase 2 with tissue inhibitor of metalloproteinase 2, Proc. Natl. Acad. Sci. U. S. A. 99 (11) (2002) 7414–7419.
[43] S. Higashi, K. Miyazaki, Identification of a region of beta-amyloid precursor protein essential for its gelatinase A inhibitory activity, J. Biol. Chem. 278 (16) (2003) 14020–14028.
[44] J.D. Mott, C.L. Thomas, M.T. Rosenbach, K. Takahara, D.S. Greenspan, M.J. Banda, Post-translational proteolytic processing of procollagen C-terminal proteinase enhancer releases a metalloproteinase inhibitor, J. Biol. Chem. 275 (2) (2000) 1384–1390.
[45] J. Oh, R. Takahashi, S. Kondo, A. Mizoguchi, E. Adachi, R.M. Sasahara, S. Nishimura, Y. Imamura, H. Kitayama, D.B. Alexander, C. Ide, T.P. Horan, T. Arakawa, H. Yoshida, S. Nishikawa, Y. Itoh, M. Seiki, S. Itohara, C. Takahashi, M. Noda,

The membrane-anchored MMP inhibitor RECK is a key regulator of extracellular matrix integrity and angiogenesis, Cell 107 (6) (2001) 789–800.
[46] D.E. Kleiner, W.G. Stetler-Stevenson, Quantitative zymography: detection of picogram quantities of gelatinases, Anal. Biochem. 218 (2) (1994) 325–329.
[47] J. Vandooren, N. Geurts, E. Martens, P.E. Van den Steen, G. Opdenakker, Zymography methods for visualizing hydrolytic enzymes, Nat. Methods 10 (3) (2013) 211–220.
[48] S.J. George, J.L. Johnson, In situ zymography, Methods Mol. Biol. 622 (2010) 271–277.
[49] J. Zhang, L. Nie, M. Razavian, M. Ahmed, L.W. Dobrucki, A. Asadi, D.S. Edwards, M. Azure, A.J. Sinusas, M.M. Sadeghi, Molecular imaging of activated matrix metalloproteinases in vascular remodeling, Circulation 118 (19) (2008) 1953–1960.
[50] W.M. Frederiks, O.R. Mook, Metabolic mapping of proteinase activity with emphasis on in situ zymography of gelatinases: review and protocols, J. Histochem. Cytochem. 52 (6) (2004) 711–722.
[51] M. Gawlak, T. Gorkiewicz, A. Gorlewicz, F.A. Konopacki, L. Kaczmarek, G.M. Wilczynski, High resolution in situ zymography reveals matrix metalloproteinase activity at glutamatergic synapses, Neuroscience 158 (1) (2009) 167–176.
[52] A. Kohrmann, U. Kammerer, M. Kapp, J. Dietl, J. Anacker, Expression of matrix metalloproteinases (MMPs) in primary human breast cancer and breast cancer cell lines: new findings and review of the literature, BMC Cancer 9 (2009) 188.
[53] C.R. Ban, S.M. Twigg, B. Franjic, B.A. Brooks, D. Celermajer, D.K. Yue, S.V. McLennan, Serum MMP-7 is increased in diabetic renal disease and diabetic diastolic dysfunction, Diabetes Res. Clin. Pract. 87 (3) (2010) 335–341.
[54] E.M. Wilson, H.R. Gunasinghe, M.L. Coker, P. Sprunger, D. Lee-Jackson, B. Bozkurt, A. Deswal, D.L. Mann, F.G. Spinale, Plasma matrix metalloproteinase and inhibitor profiles in patients with heart failure, J. Card. Fail. 8 (6) (2002) 390–398.
[55] I. Tency, H. Verstraelen, I. Kroes, G. Holtappels, B. Verhasselt, M. Vaneechoutte, R. Verhelst, M. Temmerman, Imbalances between matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) in maternal serum during preterm labor, PLoS One 7 (11) (2012) e49042.
[56] J. Helmersson-Karlqvist, T. Akerfeldt, L. Gunningberg, C.L. Swenne, A. Larsson, Serum MMP-9 and TIMP-1 concentrations and MMP-9 activity during surgery-induced inflammation in humans, Clin. Chem. Lab. Med. 50 (6) (2012) 1115–1119.
[57] G.P. Tarr, M.J. Williams, L.V. Phillips, A.M. van Rij, G.T. Jones, Pro-MMP-9/TIMP-1 ratio correlates poorly with a direct assessment of MMP-9 activity, Clin. Biochem. 44 (17–18) (2011) 1480–1482.
[58] C. Colotti, V. Angeli, S. Del Ry, M. Maltinti, S. Vittorini, D. Giannessi, Matrix metalloprotease-2 and -9 concentration and activity in serum and culture medium samples: a methodological reappraisal, Clin. Chem. Lab. Med. 45 (10) (2007) 1292–1298.
[59] G.E. Healthcare, Matrix Metalloproteinase-3 (MMP-3) Biotrak Activity Assay System, 2006, Product Booklet Code: RPN2639.
[60] G.E. Healthcare, Matrix Metalloproteinase-9 (MMP-9) Biotrak Activity Assay System, 2006, Product Booklet Code: RPN2634.
[61] G.T. Jones, G.P. Tarr, L.V. Phillips, G.T. Wilkins, A.M. van Rij, M.J. Williams, Active matrix metalloproteinases 3 and 9 are independently associated with coronary artery in-stent restenosis, Atherosclerosis 207 (2) (2009) 603–607.
[62] J.W. Chu, G.T. Jones, G.P. Tarr, L.V. Phillips, G.T. Wilkins, A.M. van Rij, M.J. Williams, Plasma active matrix metalloproteinase 9 associated to diastolic dysfunction in patients with coronary artery disease, Int. J. Cardiol. 147 (2) (2011) 336–338.
[63] G.T. Jones, I.P. Kay, J.W. Chu, G.T. Wilkins, L.V. Phillips, M. McCormick, A.M. van Rij, M.J. Williams, Elevated plasma active matrix metalloproteinase-9 level is associated

with coronary artery in-stent restenosis, Arterioscler. Thromb. Vasc. Biol. 26 (7) (2006) e121–e125.
[64] D.D. Bonnema, C.S. Webb, W.R. Pennington, R.E. Stroud, A.E. Leonardi, L.L. Clark, C.D. McClure, L. Finklea, F.G. Spinale, M.R. Zile, Effects of age on plasma matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs), J. Card. Fail. 13 (7) (2007) 530–540.
[65] M.H. Tayebjee, G.Y. Lip, K.T. Tan, J.V. Patel, E.A. Hughes, R.J. MacFadyen, Plasma matrix metalloproteinase-9, tissue inhibitor of metalloproteinase-2, and CD40 ligand levels in patients with stable coronary artery disease, Am. J. Cardiol. 96 (3) (2005) 339–345.
[66] S. Bellosta, D. Via, M. Canavesi, P. Pfister, R. Fumagalli, R. Paoletti, F. Bernini, HMG-CoA reductase inhibitors reduce MMP-9 secretion by macrophages, Arterioscler. Thromb. Vasc. Biol. 18 (11) (1998) 1671–1678.
[67] M. Canavesi, N. Baldini, A. Leonardi, G. Sironi, S. Bellosta, F. Bernini, In vitro inhibitory effect of lercanidipine on cholesterol accumulation and matrix metalloproteinases secretion by macrophages, J. Cardiovasc. Pharmacol. 44 (4) (2004) 416–422.
[68] J. Evans, J.T. Powell, E. Schwalbe, I.M. Loftus, M.M. Thompson, Simvastatin attenuates the activity of matrix metalloprotease-9 in aneurysmal aortic tissue, Eur. J. Vasc. Endovasc. Surg. 34 (3) (2007) 302–303.
[69] G. Derosa, I. Ferrari, A. D'Angelo, C. Tinelli, S.A. Salvadeo, L. Ciccarelli, M.N. Piccinni, A. Gravina, F. Ramondetti, P. Maffioli, A.F. Cicero, Matrix metalloproteinase-2 and -9 levels in obese patients, Endothelium 15 (4) (2008) 219–224.
[70] A. Staack, S. Badendieck, D. Schnorr, S.A. Loening, K. Jung, Combined determination of plasma MMP2, MMP9, and TIMP1 improves the non-invasive detection of transitional cell carcinoma of the bladder, BMC Urol. 6 (2006) 19.
[71] F. Tas, D. Duranyildiz, H. Oguz, H. Camlica, V. Yasasever, E. Topuz, Circulating levels of vascular endothelial growth factor (VEGF), matrix metalloproteinase-3 (MMP-3), and BCL-2 in malignant melanoma, Med. Oncol. 25 (4) (2008) 431–436.
[72] K.H. Boeker, C.I. Haberkorn, D. Michels, P. Flemming, M.P. Manns, R. Lichtinghagen, Diagnostic potential of circulating TIMP-1 and MMP-2 as markers of liver fibrosis in patients with chronic hepatitis C, Clin. Chim. Acta 316 (1–2) (2002) 71–81.
[73] B.L. Gruber, D. Sorbi, D.L. French, M.J. Marchese, G.J. Nuovo, R.R. Kew, L.A. Arbeit, Markedly elevated serum MMP-9 (gelatinase B) levels in rheumatoid arthritis: a potentially useful laboratory marker, Clin. Immunol. Immunopathol. 78 (2) (1996) 161–171.
[74] J.T. Peterson, H. Li, L. Dillon, J.W. Bryant, Evolution of matrix metalloprotease and tissue inhibitor expression during heart failure progression in the infarcted rat, Cardiovasc. Res. 46 (2) (2000) 307–315.
[75] F.G. Spinale, Myocardial matrix remodeling and the matrix metalloproteinases: influence on cardiac form and function, Physiol. Rev. 87 (4) (2007) 1285–1342.
[76] B. Zeng, A. Prasan, K.C. Fung, V. Solanki, D. Bruce, S.B. Freedman, D. Brieger, Elevated circulating levels of matrix metalloproteinase-9 and -2 in patients with symptomatic coronary artery disease, Intern. Med. J. 35 (6) (2005) 331–335.
[77] M.J. Hobeika, R.W. Thompson, B.E. Muhs, P.C. Brooks, P.J. Gagne, Matrix metalloproteinases in peripheral vascular disease, J. Vasc. Surg. 45 (4) (2007) 849–857.
[78] K. Jung, Matrix metalloproteinase-8 and tissue inhibitor of metalloproteinase-1 in serum do not reflect the analytes circulating in blood, Arterioscler. Thromb. Vasc. Biol. 28 (3) (2008) e15–e16, author reply e17.
[79] F. Mannello, Serum or plasma samples? The "Cinderella" role of blood collection procedures: preanalytical methodological issues influence the release and activity of circulating matrix metalloproteinases and their tissue inhibitors, hampering diagnostic

trueness and leading to misinterpretation, Arterioscler. Thromb. Vasc. Biol. 28 (4) (2008) 611–614.
[80] F. Mannello, Effects of blood collection methods on gelatin zymography of matrix metalloproteinases, Clin. Chem. 49 (2) (2003) 339–340.
[81] D. Rouy, I. Ernens, C. Jeanty, D.R. Wagner, Plasma storage at −80 degrees C does not protect matrix metalloproteinase-9 from degradation, Anal. Biochem. 338 (2) (2005) 294–298.
[82] G.P. Tarr, M.J. Williams, L.V. Phillips, A.M. van Rij, G.T. Jones, Seasonal variation and stability of matrix metalloproteinase-9 activity and tissue inhibitor of matrix metalloproteinase-1 with storage at −80 degrees C, Clin. Biochem. 44 (16) (2011) 1346–1348.
[83] F. Mannello, J.E. Tanus-Santos, C.A. Meschiari, G.A. Tonti, Differences in both matrix metalloproteinase 9 concentration and zymographic profile between plasma and serum with clot activators are due to the presence of amorphous silica or silicate salts in blood collection devices, Anal. Biochem. 374 (1) (2008) 56–63.
[84] G.S. Makowski, M.L. Ramsby, Use of citrate to minimize neutrophil matrix metalloproteinase-9 in human plasma, Anal. Biochem. 322 (2) (2003) 283–286.
[85] F. Mannello, K. Jung, G.A. Tonti, F. Canestrari, Heparin affects matrix metalloproteinases and tissue inhibitors of metalloproteinases circulating in peripheral blood, Clin. Biochem. 41 (18) (2008) 1466–1473.
[86] R.F. Gerlach, J.A. Uzuelli, C.D. Souza-Tarla, J.E. Tanus-Santos, Effect of anticoagulants on the determination of plasma matrix metalloproteinase (MMP)-2 and MMP-9 activities, Anal. Biochem. 344 (1) (2005) 147–149.
[87] G.P. Tarr, M.J. Williams, G.T. Wilkins, V.H. Chen, L.V. Phillips, A.M. van Rij, G.T. Jones, Intra-individual changes of active matrix metalloproteinase-9 are associated with clinical in-stent restenosis of bare metal stents, Cardiology 124 (1) (2013) 28–35.
[88] K. Kisand, I. Kerna, J. Kumm, H. Jonsson, A. Tamm, Impact of cryopreservation on serum concentration of matrix metalloproteinases (MMP)-7, TIMP-1, vascular growth factors (VEGF) and VEGF-R2 in Biobank samples, Clin. Chem. Lab. Med. 49 (2) (2011) 229–235.
[89] C.D. Souza-Tarla, J.A. Uzuelli, A.A. Machado, R.F. Gerlach, J.E. Tanus-Santos, Methodological issues affecting the determination of plasma matrix metalloproteinase (MMP)-2 and MMP-9 activities, Clin. Biochem. 38 (5) (2005) 410–414.

INDEX

Note: Page numbers followed by "*f*" indicate figures and "*t*" indicate tables.

C

N

O

P

R

S

T

V

W