Biomarkers in Disease:
Methods, Discoveries and Applications
Series Editor: Victor R. Preedy

Vinood B. Patel
Victor R. Preedy *Editors*

Biomarkers in Cardiovascular Disease

Springer Reference

Biomarkers in Disease: Methods, Discoveries and Applications

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In the past decade there has been a sea change in the way disease is diagnosed and investigated due to the advent of high throughput technologies, such as microarrays, lab on a chip, proteomics, genomics, lipomics, metabolomics, etc. These advances have enabled the discovery of new and novel markers of disease relating to autoimmune disorders, cancers, endocrine diseases, genetic disorders, sensory damage, intestinal diseases, etc. In many instances these developments have gone hand in hand with the discovery of biomarkers elucidated via traditional or conventional methods, such as histopathology or clinical biochemistry. Together with microprocessor-based data analysis, advanced statistics and bioinformatics these markers have been used to identify individuals with active disease or pathology as well as those who are refractory or have distinguishing pathologies. Unfortunately techniques and methods have not been readily transferable to other disease states and sometimes diagnosis still relies on single analytes rather than a cohort of markers. Furthermore, the discovery of many new markers have not been put into clinical practice, partly because of their cost and partly because some scientists are unaware of their existence or the evidence is still at the preclinical stage. In some cases the work needs further scientific scrutiny. There is thus a demand for a comprehensive and focused evidenced-based text and scientific literature that addresses these issues. Hence the formulation of *Biomarkers in Disease*: *Methods, Discoveries and Applications*. The series covers a wide number of areas including for example, nutrition, cancer, endocrinology, cardiology, addictions, immunology, birth defects, genetics and so on. The chapters are written by national or international experts and specialists.

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Vinood B. Patel • Victor R. Preedy
Editors

Biomarkers in Cardiovascular Disease

With 213 Figures and 138 Tables

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ISBN 978-94-007-7677-7 ISBN 978-94-007-7678-4 (eBook)
ISBN 978-94-007-7679-1 (print and electronic bundle)
DOI 10.1007/978-94-007-7678-4

Library of Congress Control Number: 2016939600

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer Science+Business Media B.V. Dordrecht

Volume Preface

In the present volume, ***Biomarkers in Cardiovascular Disease***, we have sections on

- **General Aspects**
- **Circulating and Body Fluid Biomarkers**
- **Specific Diseases and Conditions**
- **Molecular, Cellular, and Histological Variables**
- **Functional and Structural Variables**

While the Editors recognize the difficulties in assigning particular chapters to particular sections, the book has enormously wide coverage and includes the following areas, analytes, and conditions: testing pharmacological profiles, multiple biomarkers, use in functional foods, the extracellular matrix and collagen, PCSK9, vasoactive peptide urotensin II, fetuin-A, cholinesterase, triglycerides, high density lipoprotein-c, heart-type fatty acid binding protein (H-FABP), uncarboxylated matrix Gla protein, microRNAs, troponin, vascular endothelial growth factor-1, macrophage metalloprotease (MMP)-12, homocysteine, neutrophil gelatinase associated lipocalin (NGAL), testosterone and dihydrotestosterone, leukotrienes, 8-isoprostane, irisin, adiponectin, lipids and lipoproteins, gamma glutamyltransferase (GGT), plasma factor VIII levels, RhoA/Rho-associated kinase, polymorphisms in the vitamin D pathway, nitric oxide regulating proteins, genomics and proteomics, stem cells, virtual histology (VH), coronary plaque composition, pulse pressure and pulse pressure amplification, ventricular activation time, neutrophils, computed tomography, histology, blood flow velocity, myocardial blood, cerebral blood flow, functional transcranial Doppler ultrasound, epicardial fat thickness, electrocardiographic markers, J wave and fragmented QRS formation, intravascular ultrasound, and magnetic resonance, atrial fibrillation, chronic heart failure, abdominal aortic aneurysm, arrhythmias, resynchronization therapy, venous thromboembolism, carotid artery stenting, coronary artery disease, sudden cardiac death, diabetes, cirrhosis, and portal hypertension.

There are also many other analytes and conditions described within this volume.

Finally, the last chapter is devoted to locating resource material for biomarker discovery and applications.

The chapters are written by national or international experts and specialist. This book is specifically designed for clinical biochemists, cardiologists, cardiovascular health scientists, epidemiologists, and doctors and nurses, from students to practioners at the higher level. It is also designed to be suitable for lecturers and teachers in health care and libraries as a reference guide.

April 2015 Vinood B. Patel
London Victor R. Preedy

Series Preface

In the past decade, there has been a sea change in the way disease is diagnosed and investigated due to the advent of high-throughput technologies and advances in chemistry and physics, leading to the development of microarrays, lab-on-a-chip, proteomics, genomics, lipomics, metabolomics, etc. These advances have enabled the discovery of new and novel markers of disease relating to autoimmune disorders, cancers, endocrine diseases, genetic disorders, sensory damage, intestinal diseases, and many other conditions too numerous to list here. In many instances, these developments have gone hand in hand with the discovery of biomarkers elucidated via traditional or conventional methods, such as histopathology, immunoassays, or clinical biochemistry. Together with microprocessor-based data analysis, advanced statistics, and bioinformatics these markers have been used to identify individuals with active disease as well as those who are refractory or have distinguishing pathologies.

Unfortunately, techniques and methods have not been readily transferable to other disease states, and sometimes diagnosis still relies on a single analyte rather than a cohort of markers. Furthermore, the discovery of many new markers has not been put into clinical practice partly because of their cost and partly because some scientists are unaware of their existence or the evidence is still at the preclinical stage. There is thus a demand for a comprehensive and focused evidenced-based text and scientific literature that addresses these issues. Hence the book series **Biomarkers in Disease: Methods, Discoveries and Applications**. It imparts holistic information on the scientific basis of health and biomarkers and covers the latest knowledge, trends, and treatments. It links conventional approaches with new platforms. The ability to transcend the intellectual divide is aided by the fact that each chapter has:

- ***Key Facts (areas of focus explained for the lay person)***
- ***Definitions of Words and Terms***
- ***Potential Applications to Prognosis, Other Diseases, or Conditions***
- ***Summary Points***

The material in ***Potential Applications to Prognosis, Other Diseases, or Conditions*** pertains to speculative or proposed areas of research, cross-transference to

other diseases or stages of the disease, translational issues, and other areas of wide applicability.

The Series is expected to prove useful for clinicians, scientists, epidemiologists, doctors, and nurses, and also academicians and students at an advanced level.

April 2015 Victor R. Preedy
London

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About the Editors

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Dr. Vinood B. Patel, **B.Sc., Ph.D., FRSC** is currently a Reader in Clinical Biochemistry at the University of Westminster and honorary fellow at King's College London. He presently directs studies on metabolic pathways involved in liver disease, particularly related to mitochondrial energy regulation and cell death. Research is being undertaken to study the role of nutrients, antioxidants, phytochemicals, iron, alcohol, and fatty acids in the pathophysiology of liver disease. Other areas of interest are identifying new biomarkers that can be used for diagnosis and prognosis of liver disease, understanding mitochondrial oxidative stress in Alzheimer's disease, and gastrointestinal dysfunction in autism. Dr. Patel graduated from the University of Portsmouth with a degree in Pharmacology and completed his Ph.D. in Protein Metabolism from King's College London in 1997. His postdoctoral work was carried out at Wake Forest University Baptist Medical School studying structural-functional alterations to mitochondrial ribosomes, where he developed novel techniques to characterize their biophysical properties. Dr. Patel is a nationally and internationally recognized liver researcher and was involved in several NIH-funded biomedical grants related to alcoholic liver disease. Dr. Patel has edited biomedical books in the area of nutrition and health prevention, autism, and biomarkers and has published over 150 articles, and in 2014 he was elected as a Fellow to The Royal Society of Chemistry.

Victor R. Preedy B.Sc., Ph.D., D.Sc., FRSB, FRSH, FRIPHH, FRSPH, FRCPath, FRSC is a senior member of King's College London (Professor of Nutritional Biochemistry) and King's College Hospital (Professor of Clinical Biochemistry; Honorary). He is attached to both the Diabetes and Nutritional Sciences Division and the Department of Nutrition and Dietetics. He is also founding and

current Director of the Genomics Centre and a member of the School of Medicine. Professor Preedy graduated in 1974 with an Honours Degree in Biology and Physiology with Pharmacology. He gained his University of London Ph.D. in 1981. In 1992, he received his Membership of the Royal College of Pathologists, and in 1993 he gained his second doctoral degree, for his contribution to the science of protein metabolism in health and disease. Professor Preedy was elected as a Fellow of the Institute of Biology (Society of Biology) in 1995 and to the Royal College of Pathologists in 2000. He was then elected as a Fellow to the Royal Society for the Promotion of Health (2004) and The Royal Institute of Public Health and Hygiene (2004). In 2009, Professor Preedy became a Fellow of the Royal Society for Public Health, and in 2012 a Fellow of the Royal Society of Chemistry. In 2015, the Society of Biology received its Royal Charter, so Professor Preedy became an FRSB. In his career, Professor Preedy worked at the National Heart Hospital (part of Imperial College London) and the MRC Centre at Northwick Park Hospital. He has collaborated with research groups in Finland, Japan, Australia, USA, and Germany. He is a leading expert on biomedical sciences and has a long-standing interest in analytical methods and biomarkers, especially their applications to the study of health and disease. He has lectured nationally and internationally. To his credit, Professor Preedy has published over 500 articles, which includes peer-reviewed manuscripts based on original research, reviews, abstracts, and numerous books and volumes.

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Part I

General Aspects

Testing Pharmacological Profiles with Biomarkers Relevant to Cardiovascular Profiles

1

Giuseppe Derosa and Pamela Maffioli

Contents

Abstract

Cardiovascular diseases are the leading cause of morbidity and mortality in the United States and in Europe. Early identification of biomarkers linked to cardiovascular disease can be effective in identifying high-risk patients to early treat them in order to reduce cardiovascular diseases. The aim of this chapter is to

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_27

examine biomarkers relevant to cardiovascular diseases in order to identify targets where pharmacological treatment can act. In this chapter, we will examine the main markers of insulin resistance, inflammation, endothelial damage, and metabolism linked to cardiovascular diseases.

Keywords

Adhesion molecules • Biomarkers • Cardiovascular diseases • Inflammation • Insulin resistance

Abbreviations

ADN	Adiponectin
Apo A-I	Apolipoprotein A-I
Apo B	Apolipoprotein B
C-IMT	Carotid intima-media thickness
DBP	Diastolic blood pressure
FPG	Fasting plasma glucose
FPI	Fasting plasma insulin
Hs-CRP	High-sensitivity C-reactive protein
IL-6	Interleukin-6
Lp(a)	Lipoprotein(a)
MMP-2	Metalloproteinase-2
MMP-9	Metalloproteinase-9
MPO	Myeloperoxidase
PAI-1	Plasminogen activator inhibitor-1
PON-1	Paraoxonase-1
PPG	Postprandial glucose
RBP-4	Retinol binding protein-4
SBP	Systolic blood pressure
sE-selectin	Soluble E-selectin
sICAM	Serum intracellular adhesion molecule-1
sVCAM	Soluble vascular cell adhesion molecule-1
TNF-α	Tumor necrosis factor-α

Key Facts of Cardiovascular Diseases

- Cardiovascular diseases include a group of conditions that involve ischemic heart disease, stroke, and peripheral artery disease.
- The underlying mechanism of cardiovascular diseases involves atherosclerosis.
- There are factors accelerating atherosclerosis; they include smoke, physical inactivity, diabetes, weight gain, elevated blood pressure, and inadequate lipid profile.
- In order to prevent atherosclerosis, early identification and correction of these risk factors is very important.

- When identified, risk factors should be corrected; in particular, we can improve lipid profile using hypocholesterolaemic agents, reduce glycaemia with antiglycaemic agents, and induce weight decrease with a well-balanced diet.

Definitions

Adipocytokines A family of bioactive products secreted by adipose tissue; adipocytokines include inflammatory mediators, angiogenic proteins, and metabolic regulators.

Atherosclerosis Atherosclerosis is a process in which an artery wall thickens as a result of chronic inflammatory response to cholesterol deposit.

Biomarker The term refers to a measurable indicator of some biological state or condition that can be used for diagnosis or follow-up a particular disease.

Endothelial dysfunction It is a systemic pathological state of the endothelium in response to cardiovascular risk factors and precedes the development of atherosclerosis.

HOMA index Is a method used to quantify insulin resistance and β-cell function; it is calculated with a formula that considers glycaemia and insulinemia.

Introduction

Cardiovascular diseases are the leading cause of morbidity and mortality in the United States and in Europe, and for this reason primary and secondary prevention of cardiovascular diseases are public health priorities (Pearson et al. 2002). In this regard, biomarkers are one tool to better identify high-risk patients, in order to promptly and accurately diagnose diseases and to effectively prognosticate and treat patients.

Biomarker has been defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Biomarkers Definitions Working Group 2001). Biomarkers may also serve as surrogate end points. A surrogate end point is one that can be used as an outcome in clinical trials to evaluate safety and effectiveness of therapies instead of measurement of the true outcome of interest. The underlying principle is that alterations in the surrogate end point track closely with changes in the outcome of interest (Colburn 2000; De Gruttola et al. 2001). Surrogate end points have the advantage that they may be gathered in a shorter time frame and with less expense than end points such as morbidity and mortality, which require large clinical trials for evaluation.

In this regard, the aim of this chapter is to examine biomarkers relevant to cardiovascular disease in order to identify targets where pharmacological treatment can act in order to give practical considerations useful for the clinical practice.

Description of the Main Biomarkers Relevant to Cardiovascular Profiles

Markers of Insulin Resistance

Insulin resistance is a hallmark of obesity, diabetes, and cardiovascular diseases and leads to many of the abnormalities associated with metabolic syndrome. Insulin resistance is established by genetic and environmental factors. Insulin resistance leads to impaired glucose tolerance and plays an important pathophysiological role in the development of diabetes (DeFronzo et al. 1992). Patients with insulin resistance are likely to have impaired fasting plasma glucose levels, which, in turn, enhance the prevalence of more atherogenic, small dense low-density lipoprotein (LDL) particles. Central obesity and insulin resistance form the basis of the pathophysiology of dyslipidaemia, lack of glucose tolerance, and the existence of chronic subclinical inflammation and hypertension in metabolic syndrome.

The markers of insulin resistance are listed in Table 1 and include:

- *Bioumoral markers*: glycaemia, fasting plasma insulin, HOMA index, small and dense LDL
- *Adipocytokines*: adiponectin, resistin, visfatin, vaspin, retinol binding protein-4 (RBP-4)
- *Fat*: subcutaneous fat, visceral fat, epicardial fat

Bioumoral Markers

The quantification of insulin resistance can be performed by evaluating the peripheral insulin sensitivity in vivo with methods such as the pancreatic suppression test (Greenfield et al. 1981), the hyperinsulinemic-euglycaemic clamp technique (Greenfield et al. 1981), and the minimal model approximation of the metabolism of glucose (MMAMG) (Bergman et al. 1985). They are complicated, time-consuming, and expensive methods suitable only for studies with a small number of subjects. For epidemiologic and clinical studies, simpler, indirect methods have been advocated for quantification of insulin resistance, based on measuring plasma insulin levels during fasting or after glucose stimulus and on the insulin-glucose ratio calculated with different mathematical formulas. Such methods include measurement of fasting plasma insulin levels and 2-h post-75-g oral glucose load, the homeostasis model assessment (HOMA) (Matthews et al. 1985), and the mathematical calculations known as the quantitative insulin sensitivity check index (QUICKI) (Katz et al. 2000) and McAuley et al. (2001) indexes. Among these indexes, the HOMA index has been validated with the hyperinsulinemic-euglycaemic clamp technique (Bonora et al. 2000), and, therefore, it is considered a valid method to assess

Table 1 Markers of insulin resistance

Bioumoral markers	Adipocytokines	Fat
Glycaemia	Adiponectin	Subcutaneous fat
Fasting plasma insulin	Resistin	Visceral fat
HOMA index	Visfatin	Epicardial fat
Small and dense LDL	Vaspin	
	Retinol binding protein-4	

HOMA Index = [fasting plasma glucose (mg/dl) x fasting plasma insulin (μU/ml)]/405

or

HOMA Index = [fasting plasma glucose (mmol/l) x fasting plasma insulin (mU/l)]/22.5

QUICKI index = 1/[log(fasting plasma insulin (μU/mL) + log(fasting plasma glucose (mg/dL)]

McAuley index = exp[2.63-0.28 ln(fasting plasma insulin (mU/l)) - 0.31 ln(triglycerides (mmol/l))]

Fig. 1 Formulas to calculate indexes of insulin resistance

peripheral insulin sensitivity in epidemiologic studies (Ascaso et al. 2003). To see how these indexes can be calculated, see Fig. 1.

Regarding small and dense LDL, an increased concentration of LDL cholesterol is widely recognized as a risk factor for coronary heart disease (Castelli et al. 1986). There is considerable heterogeneity in the size and density of LDL particles (Shen et al. 1981). Austin et al. (1988) found that most individuals can be assigned to one of two LDL subclass patterns (A or B). Small, dense LDL particles (pattern B) are thought to be more atherogenic than larger LDL particles. Reaven et al. (1993) showed that subjects with a preponderance of small, dense LDL particles (pattern B) were more insulin resistant and had higher triglyceride concentrations than subjects with larger LDL particles (pattern A). This was confirmed also by Mykkänen et al. that concluded that a preponderance of small, dense LDL particles is associated with insulin resistance and that serum triglycerides concentration modifies this relationship (Mykkänen et al. 1997), implying that triglycerides levels are a contributing factor to insulin resistance.

Adipocytokines

It is now clear that adipose tissue is a complex and highly active metabolic and endocrine organ. Besides adipocytes, adipose tissue contains connective tissue matrix, nerve tissue, stromovascular cells, and immune cells. Although adipocytes

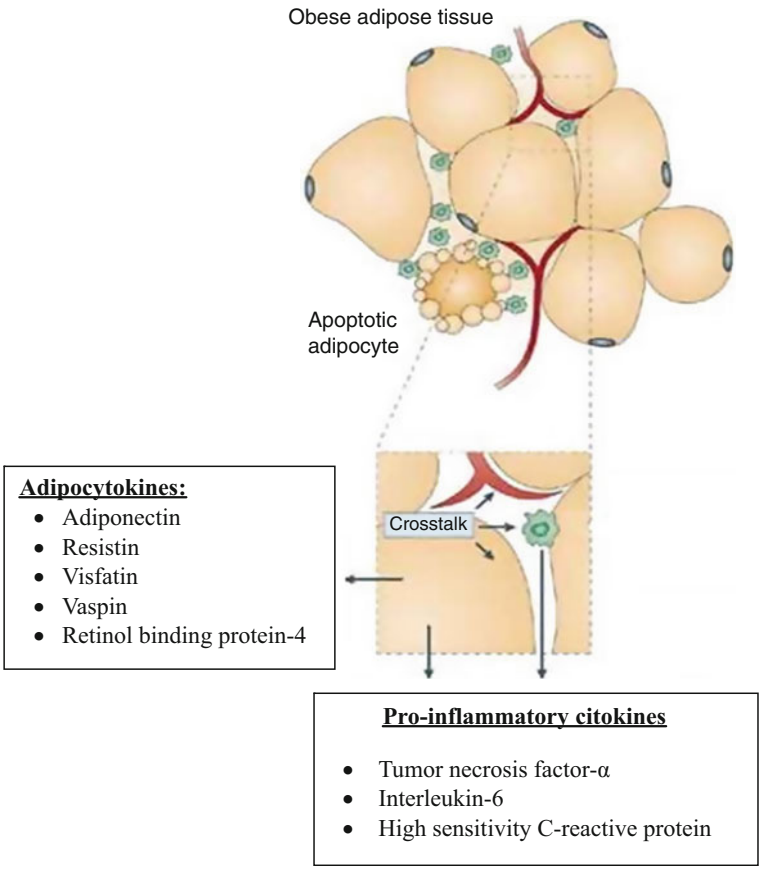


Fig. 2 Mechanism of insulin resistance. (Adapted from Tilg et al. 2006).

express and secrete several endocrine hormones such as leptin and adiponectin, many secreted proteins are derived from the non-adipocyte fraction of adipose tissue. Regardless, these components function as an integrated unit, making adipose tissue a true endocrine organ (Fig. 2).

Adiponectin is a protein exclusively synthesized by adipocytes; it is decreased in obesity and inversely related to glucose and insulin. Ablation of the adiponectin gene in mice resulted in insulin resistance, glucose intolerance, dyslipidaemia, and increased susceptibility to vascular injury and atherosclerosis (Berg et al. 2002). Adiponectin reverses these abnormalities by stimulating oxidation of fatty acids, suppressing gluconeogenesis and inhibiting monocyte adhesion, macrophage transformation, and proliferation and migration of smooth muscle cells in blood vessels (Berg et al. 2002).

Human resistin, instead, is secreted by mononuclear cells and activated macrophages (Steppan et al. 2001) and was named for its ability to induce insulin resistance in rodents. Resistin has been linked to obesity and diabetes, and it has

been reported to be elevated in adipose tissue and serum in obesity and insulin resistance (Azuma et al. 2004). Moreover, resistin appears to confer an increased risk of inflammation and atherosclerosis (Silswal et al. 2005).

Visfatin was discovered as a secretory protein highly enriched in rodent and human visceral adipocytes (Fukuhara et al. 2005). The expression and secretion of visfatin is increased during the development of obesity; however, in contrast with inflammatory cytokines, the rise in visfatin does not decrease insulin sensitivity. Instead, visfatin exerts insulin-mimetic effects in cultured adipocytes, hepatocytes, and myotubes and lowers plasma glucose in mice. Visfatin binds to the insulin receptor with similar affinity but at a site distinct from insulin (Fukuhara et al. 2005). In contrast with insulin, visfatin levels do not change with feeding and fasting (Fukuhara et al. 2005). The discovery of visfatin may lead to novel therapies for diabetes; however, it remains to be determined if visfatin acts in concert with insulin to regulate metabolism and whether such interaction occurs via endocrine or paracrine mechanisms. Vaspin (visceral adipose tissue-derived serpin) is a member of the serine protease inhibitor family isolated by from the visceral white adipose tissue of OLETF (Otsuka Long-Evans Tokushima fatty) rats, a model of abdominal obesity, insulin resistance, and diabetes (Hida et al. 2005). Administration of vaspin to obese mice improves glucose tolerance and insulin sensitivity and reverses the expression of half of the number of genes induced in white adipose tissue by diet-induced obesity (Hida et al. 2005).

Finally, RBP-4, the sole retinol transporter in blood, is secreted from adipocytes and liver. Serum RBP-4 levels correlate highly with insulin resistance, other metabolic syndrome factors, and cardiovascular disease. Elevated RBP-4 levels cause insulin resistance in mice and humans (Yang et al. 2005), but the molecular mechanisms are unknown. Probably RBP-4 induces expression of proinflammatory cytokines in mouse and human macrophages and, thereby, indirectly inhibits insulin signaling in cocultured adipocytes. Studies in mice suggest that adipose tissue serves as a glucose sensor and regulates systemic glucose metabolism through release of RBP-4 in response to decreased intracellular glucose concentrations (Yang et al. 2005).

Fat

Fat accumulates mainly in subcutaneous depots, but sizeable amounts of adipose tissue are also deposited in the abdomen (between and within organs), in the thorax (as epicardial, mediastinal and/or intramyocardial fat), in the pancreas, and in skeletal muscle. Although the impact of central versus peripheral body fat on both metabolic and cardiovascular dysfunction has been firmly established, the importance of the site of abdominal fat accumulation in relation to insulin sensitivity is still a matter of some debate. Some studies have suggested that the intra-abdominal fat depot is the major determinant of insulin resistance (Canepa et al. 2013; Park et al. 1991), and of other features of the metabolic syndrome (Després, et al. 1989), whereas others have suggested that the subcutaneous fat compartment is the most critical determinant of insulin sensitivity (Mazurek et al. 2003). Several methods of assessing the amount of visceral fat accumulation have been investigated; they can be helpful to estimate not only visceral obesity but also the risk of cardiovascular and metabolic diseases.

The simplest way to assess visceral fat is to use an anthropometric index such as body mass index, waist circumference, waist hip ratio, abdominal sagittal diameter, or neck circumference. These values provide a fast, easy, and noninvasive method of assessing regional adiposity, particularly in epidemiologic studies. However, it is possible that substantial variations in the visceral fat content may be observed among persons with a similar waist circumference or waist hip ratio value, because these indexes are not the direct methods of quantifying the amount of fat or of discriminating between visceral and subcutaneous fat. Accordingly, an alternative and reliable method can be assessment of visceral fat thickness by ultrasonography. Using this technique, subcutaneous and visceral fat diameters can be measured using a 3.0 MHz curved array transducer. Visceral adipose tissue diameter can be measured from the internal surface of the rectus abdominis muscle to the near wall of the aorta. Subcutaneous adipose tissue diameter can be measured at the same position as the distance between the external surface of the muscle and the skin. The thickness of the muscle and skin need to be excluded.

Also cardiac fat is now recognized as a new cardiometabolic risk marker, as it is associated with increased insulin resistance, cardiovascular risk factors, visceral fat, and, in general, with the metabolic syndrome. In the supradiaphragmatic region, fat is deposited in the intrathoracic space (extra-pericardial adipose tissue or mediastinal fat), around the myocardium (epicardial adipose tissue) and as intramyocardial fat. The few data available in literature suggest that epicardial fat can be an important determinant of diastolic dysfunction (Abate et al. 1995). Because epicardial adipose tissue secretes proinflammatory, proatherogenic, and prothrombotic adipokines (Dutour et al. 2010) and there is no physical barrier separating it from the adjacent myocardium and coronary arteries, epicardial adipose tissue can have a local metabolic role by a paracrine effect (Sacks and Fain 2007).

Inflammatory Markers

Several older prospective epidemiological studies have documented an association between inflammatory markers such as white blood cells count and fibrinogen (Yarnell et al. 1991) and cardiovascular disease. Since these publications, a large number of peer-reviewed scientific reports have been published relating inflammatory markers to cardiovascular disease. In the latest years, several commercial assays to assess inflammatory markers have become available. As a consequence of the expanding research base and availability of assays, the number of inflammatory marker tests ordered by clinicians for cardiovascular disease risk prediction has grown rapidly.

The inflammatory markers are listed in Table 2, and include:

- *Bioumoral markers*: white blood cells, platelets, fibrinogen.
- *Cytokines*: tumor necrosis factor-α (TNF-α), high-sensitivity C-reactive protein (Hs-CRP), myeloperoxidase (MPO), interleukin-6 (IL-6), paraoxonase-1 (PON-1).

Table 2 Inflammatory markers

Bioumoral markers	Cytokines
White blood cells	Tumor necrosis factor-α
Platelets	High-sensitivity C-reactive protein
Fibrinogen	Myeloperoxidase
	Interleukin-6
	Paraoxonase-1

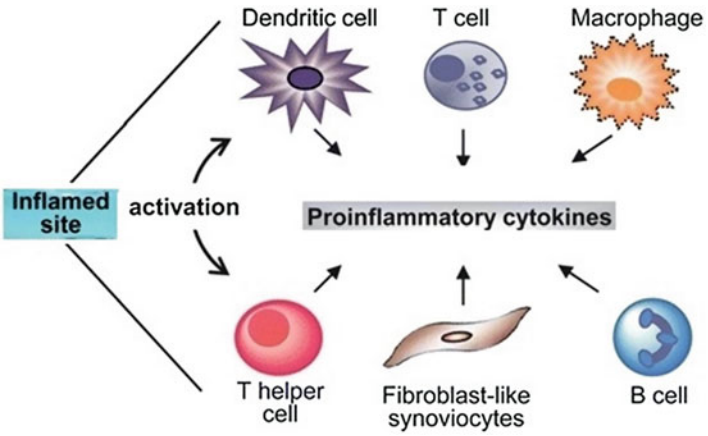


Fig. 3 Mechanism of inflammation. (Adapted from Pan et al. 2010).

Bioumoral Markers

An elevated total white blood cell count is a risk factor for atherosclerotic vascular disease. White blood cell-derived macrophages and other phagocytes are believed to contribute to vascular injury and atherosclerotic progression (Fuster and Lewis 1994) (Fig. 3). Several prospective studies have shown a positive and independent association between white blood cell count and coronary heart disease incidence or mortality (Folsom et al. 1997; de Labry et al. 1990). The presence of leukocytes within atherosclerotic arteries was reported in the early 1980s. Initially, investigators thought that only macrophages were predominantly present within atherosclerotic vessels; however, several studies reported the presence of most known leukocytes in both mouse and human aortas. A key initiating process of atherosclerosis is the intimal retention of apolipoprotein B (Apo B) containing lipoproteins in regions of disturbed blood flow and low shear stress. In response to intimal lipid accumulation, disturbed blood flow, low shear stress, and other stimuli, endothelial cells permit monocytes, major precursors of macrophages, passage across the endothelium. Newly-infiltrated monocyte-derived macrophages recognize and ingest lipids that have accrued in the intima as a consequence of hypercholesterolemia. Macrophages are specialized phagocytes that rely on different strategies to sense, internalize, and process the diverse lipid moieties they encounter. Lipoprotein recognition and consequent ingestion morphs macrophages into lipid-rich macrophages, known as

foam cells, many of which eventually die and contribute to a large lipid core, a characteristic of lesions most vulnerable to rupture. In experimental atherosclerosis, neutrophils, whose numbers rise in the blood, help monocyte adhesion or transmigration by releasing alarmins and other preformed granular proteins. Neutrophils also contain large quantities of myeloperoxidase, NADPH oxidase, and lipoxygenases, which contribute to oxidative stress, a major determinant of endothelial cell dysfunction, lesion growth, and instability of plaque. Also mast cells promote atherosclerosis by releasing the contents of their protease-cytokine-autacoid-rich granules.

Platelets also play an important role. In normal situations, platelets can detect a disruption in the lining of a blood vessel and react to build a wall to stop bleeding. In cardiovascular disease, abnormal clotting occurs resulting in heart attacks or stroke. During atherosclerosis, blood vessels injured by smoking, cholesterol, or high blood pressure develop cholesterol-rich plaques that line the blood vessel; these plaques can rupture and cause the platelets to form a clot (Gregg and Goldschmidt-Clermont 2003). During plaque rupture, they form the thrombus that causes ischemia of downstream tissue in heart attacks, strokes, and peripheral vascular disease.

Fibrinogen is a protein produced by the liver; it usually helps stop bleeding by helping blood clots to form. However, fibrinogen has been identified as an independent risk factor for cardiovascular disease and associated with traditional cardiovascular risk factors (Lee et al. 1993), suggesting that elevation of fibrinogen may be a pathway by which these risk factors exert their effect. There are several mechanisms by which fibrinogen may increase cardiovascular risk. First, it binds specifically to activated platelets via glycoprotein IIb/IIIa, contributing to platelet aggregation. Second, increased fibrinogen levels promote fibrin formation. Third, fibrinogen is a major contributor to plasma viscosity. Finally, it is an acute-phase reactant that is increased in inflammatory states (Stec et al. 2000).

Cytokines

Among many inflammatory markers, TNF-α emerged as a key cytokine that influences intermediary metabolism. TNF-α is a cell signaling protein involved in systemic inflammation and is one of the cytokines that make up the acute phase reaction. It is produced chiefly by activated macrophages, although it can be produced by many other cell types such as monocytes, T-cells, smooth muscle cells, adipocytes, and fibroblasts. Tumor necrosis factor-α has important proinflammatory properties, which play crucial roles in the innate and adaptive immunity, cell proliferation, and apoptotic processes (Popa et al. 2007). Tumor necrosis factor-α also plays a crucial role in the development of atherosclerotic lesions. In addition, TNF-α is able to induce proatherogenic lipoprotein changes. Finally, TNF-α, by decreasing insulin sensitivity, contributes to the development of glucose metabolism disturbances (Popa et al. 2007).

As already said above, inflammation plays a major role in the initiation, progression, and destabilization of atheromas. Laboratory and experimental evidence indicate that atherosclerosis, in addition to being a disease of lipid accumulation, also represents a chronic inflammatory process. Thus, researchers have hypothesized that

inflammatory markers such as Hs-CRP may provide an adjunctive method for global assessment of cardiovascular risk. High-sensitivity C-reactive protein is an acute-phase reactant, produced predominantly in hepatocytes as a pentamer of identical subunits in response to several cytokines; it is a circulating acute-phase reactant that reflects active systemic inflammation. An association of Hs-CRP with risk for cardiovascular disease has been described in many studies, the Multiple Risk Factor Intervention Trial was the first of many primary prevention, prospective epidemiological studies to show a strong relationship between levels of Hs-CRP and mortality from coronary heart disease in high-risk middle-aged men (Kuller et al. 1996).

High-sensitivity C-reactive protein rises acutely after tissue injury, including myocardial infarction. Intense cytokine production and inflammatory cell infiltration occurs in the area of ischemia and necrosis. This increase in Hs-CRP, in part, correlates with infarct size and with a higher risk of cardiac rupture.

Myeloperoxidase is a leukocyte-derived enzyme that catalyzes the formation of a number of reactive oxidant species. In addition to being an integral component of the innate immune response, evidence has emerged that MPO-derived oxidants contribute to tissue damage during inflammation and atherosclerosis. Myeloperoxidase has been linked to activation of protease cascades and both proapoptotic and prothrombotic pathways that are believed to be involved in plaque fissuring development of superficial erosions and intracoronary thrombus generation during sudden cardiac death (Fu et al. 2001; Baldus et al. 2004).

Increased levels of IL-6 have been also associated with high risk of all-cause mortality in older people. Interleukin-6 plays an important role in mediating inflammation and is a central stimulus for the acute-phase response (Papanicolaou et al. 1998). In particular, IL-6 induces the hepatic synthesis of C-reactive protein (CRP), described above as a known proinflammatory marker of atherothrombotic vascular disease.

Serum PON-1 is an HDL-associated lipo-lactonase, which is synthesized and secreted by the liver. Several lines of clinical and experimental evidence strongly support a potential role for PON-1 in protection against atherosclerosis. Paraoxonase-1 has anti-atherogenic properties, which are associated with the enzyme's capability to protect LDL, as well as HDL from oxidation, to decrease macrophage oxidative status, to stimulate cholesterol efflux from macrophages, and to decrease oxidative status in atherosclerotic lesions. Furthermore, PON-1 was suggested to contribute to the anti-inflammatory activity of HDL by destroying biologically active lipids in mildly oxidized LDL, resulting in decreased inflammatory responses in the artery wall cells. Moreover, PON-1 was shown to decrease monocyte chemotaxis and adhesion to endothelial cells, to inhibit monocyte-to-macrophage differentiation, and the absence of PON-1 was shown to be associated with overexpression of adhesion molecules (Aharoni et al. 2013). Experiments with transgenic PON-1 knockout mice confirm the potential for PON-1 to protect against atherogenesis. Oxidation of serum low density lipoproteins (LDL) is an important early step in the development of atherosclerosis. The oxidized products are scavenged by macrophages which eventually transform into foam cells, filled with cholesterol esters. They eventually become fatty streaks in the endothelium and

Table 3 Endothelial damage markers

Bioumoral markers	Cellular adhesion molecules	Matrix metalloproteinases	Clinical markers
Postprandial dyslipidaemia	Soluble intracellular adhesion molecule-1	Metalloproteinases-2	Systolic and diastolic blood pressure
Microalbuminuria	Soluble vascular cell adhesion molecule-1	Metalloproteinases-9	Carotid intima-media thickness
Nitrites/nitrates	sE-selectin		
Plasminogen activator inhibitor-1			

finally are seen as atheromatous plaques. Mackness was the first to suggest that serum PON-1 may be able to protect against the initial stage of this process, the oxidation of the LDL phospholipids (La Du et al. 1999).

Endothelial Damage Markers

Endothelial dysfunction is a well established response to cardiovascular risk factors and precedes the development of atherosclerosis. Endothelial dysfunction is involved in lesion formation by the promotion of both the early and late mechanisms of atherosclerosis including upregulation of adhesion molecules, increased chemokine secretion and leukocyte adherence, increased cell permeability, enhanced low-density lipoprotein oxidation, platelet activation, cytokine elaboration, and vascular smooth muscle cell proliferation and migration. Endothelial dysfunction is a term that covers diminished production/availability of nitric oxide and/or an imbalance in the relative contribution of endothelium-derived relaxing and contracting factors (Hadi et al. 2005).

Endothelial damage markers are listed in Table 3 and include:

- *Bioumoral markers*: postprandial dyslipidaemia, microalbuminuria, nitrites/nitrates, plasminogen activator inhibitor-1 (PAI-1)
- *Cellular adhesion molecules*: soluble intracellular adhesion molecule-1 (sICAM-1), soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble E-selectin (sE-selectin)
- *Matrix metalloproteinases*: metalloproteinases-2 and -9 (MMP-2 and -9)
- *Clinical markers*: systolic and diastolic blood pressure, carotid intima-media thickness (C-IMT)

Bioumoral Markers

In people with diabetes or insulin resistance, the incidence of dyslipidaemia is high, and lipoprotein abnormalities play a key role in the development of atherosclerotic vascular complications. Increased postprandial lipidaemia is a characteristic aspect of diabetic dyslipidaemia; it has a high prevalence among people with diabetes even

when they have normal fasting triglycerides levels. Postprandial hyperlipidaemia exerts a relevant effect on the development of diabetic macrovascular complications since the excessive increment of triglycerides and their persistence in the circulation is accompanied by deviations towards a more atherogenic metabolism, with accumulation of remnants and small, dense LDL. These are more easily oxidized than large buoyant LDL (Rebolledo and Actis Dato 2005). The most adequate way to experimentally reproduce the postprandial lipemia condition appears to be the administration of a standardized oral fat load (OFL) to fasting patient. Clinical studies confirmed that OFL gave a reduction of nitrites/nitrates and ADN and increase MMP-2 and MMP-9 (Derosa et al. 2010a). At the same time, OFL induced a complex and massive systemic inflammatory response that included IL-6, TNF-α, Hs-CRP, and cell adhesion molecules, even before triglycerides significantly rose (Derosa et al. 2009).

Microalbuminuria is a strong and independent indicator of increased cardiovascular risk among individuals with and without diabetes. Therefore, microalbuminuria can be used for stratification of risk for cardiovascular disease. Once microalbuminuria is present, cardiovascular risk factor reduction should be more "aggressive" (Stehouwer and Smulders 2006). Dinneen and Gerstein (Dinneen and Gerstein 1997), in a systematic review, showed microalbuminuria among individuals with type 2 diabetes to be associated with a 2.4-fold increased risk for cardiovascular death as compared with normoalbuminuria. In addition, similar associations exist in hypertensive individuals (without diabetes) and in the general population (Jager et al. 1999). In the organism, nitrate and nitrite may function as an alternative source for nitric oxide (NO), an important and multifaceted physiological signaling molecule. Potential protective mechanisms related to cardiovascular diseases include vasodilation, inhibition of endothelial dysfunction, and inhibition of platelet aggregation. Endothelium-derived NO is an important signaling agent in the regulation of blood pressure (Tang et al. 2011). NO-mediated regulation of vascular tone involves increased cyclic guanosine monophosphate (cGMP) and subsequent relaxation of vascular smooth muscle (Tang et al. 2011). Nitric oxide suppresses systemic PAI-1 levels; elevated plasma PAI-1 levels are associated with endothelial dysfunction (Bouchie et al. 1998). Circulating PAI-1 levels are elevated in patients with type 2 diabetes, substantially contributing to the prothrombotic and proatherosclerotic changes in diabetes. In addition, plasma PAI-1 levels are elevated throughout the spectrum of insulin resistance, from the metabolic syndrome to prediabetes (period of impaired glucose tolerance) to diabetes (Pannacciulli et al. 2002).

Cellular Adhesion Molecules

Cellular adhesion molecules mediate the margination, adhesion, and transendothelial migration of circulating mononuclear cells from the blood stream to the extravascular compartment and have an important role in the progression of atherosclerotic plaque (Ross 1999). In addition, adhesion molecules and various other cytokines recruit and activate mononuclear cells to release matrix metalloproteinases, promoting plaque rupture and the initiation of acute coronary syndromes (Ross 1999).

Various inflammatory markers have been proposed to assist in the prediction of subsequent coronary events among healthy people (Ridker et al. 2000). Soluble intercellular adhesion molecule-1 and other adhesion molecules have been found to be associated with subsequent incidence of coronary heart disease among healthy men and women (Ridker et al. 2001), even if sICAM-1 was a better predictor for the presence of atherosclerosis and coronary heart disease than other adhesion molecules (Porsch-Oezçueruemez et al. 1999). Two prospective cohort studies, in fact, indicate that baseline levels of sICAM-1 are increased many years before a first myocardial infarction occurs (Ridker et al. 2000); data for sVCAM-1 are not so certain (de Lemos et al. 2000). Regarding sE-selectin, instead, it confirmed to be a reliable marker and to be strongly associated with traditional cardiovascular risk factors. E-selectin mediate leukocyte rolling on the endothelium and platelet-leukocyte interaction; it is only expressed in activated endothelial cells and acts as an adhesive reactant. On activation, sE-selectin is released into the circulation. Increased levels of sE-selectin have been found in individuals with myocardial infarction, and sE-selectin levels are related to blood pressure (Thorand et al. 2006).

Matrix Metalloproteinases

Matrix metalloproteinases are a family of proteolytic enzymes that are regulated by inflammatory signals to mediate changes in extracellular matrix. Humans have 24 matrix metalloproteinase genes including duplicated MMP-23 genes; thus there are 23 MMPs in humans. The activities of most MMPs are very low or negligible in the normal steady-state tissues, but expression is transcriptionally controlled by inflammatory cytokines, growth factors, hormones, cell-cell and cell-matrix interaction. Matrix metalloproteinases are important in vascular remodeling, not only in the overall vasculature architecture but also, more importantly, in the advancing atherosclerotic plaque. Matrix metalloproteinases activation modifies the architecture of the plaque and may directly participate in the process of plaque rupture (Liu et al. 2006). Metalloproteinases are extremely potent protein degradation and modifying enzymes; thus, their biological actions are very tightly controlled by tissue inhibitor of metalloproteinases. Among MMPs, more specifically, MMP-2 and MMP-9 play an important role in vascular remodeling (Gibbons and Dzau 1994). MMP-2 and -9 are Zn^{+2} dependent endopeptidases, synthesized and secreted in zymogen form. The nascent form of the protein shows an N-terminal signal sequence ("pre" domain) that directs the protein to the endoplasmic reticulum. The pre domain is followed by a propeptide-"pro" domain that maintains enzyme latency until cleaved or disrupted and a catalytic domain that contains the conserved zinc-binding region. A hemopexin/vitronectin-like domain is also seen, that is connected to the catalytic domain by a hinge or linker region (Figs. 4, and 5). Increased MMP-2 and MMP-9 activity is associated with destruction of the elastic laminae of arteries and aneurysm formation in animals and humans (Longo et al. 2002). Metalloproteinase-2 and −9 resulted increased in patients with obesity (Derosa et al. 2008), hypertension (Derosa et al. 2006), type 2 diabetes (Derosa et al. 2007a), acute coronary syndrome (Derosa et al. 2007b, c, d). More recently, plasma MMP-9 levels have

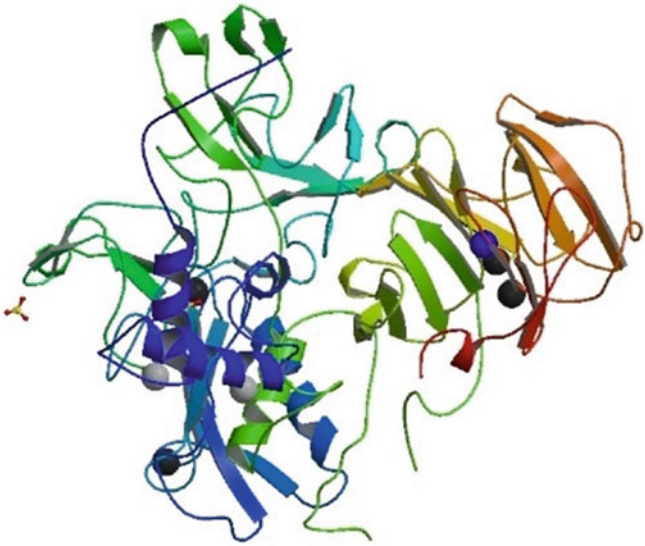


Fig. 4 MMP-2 structure

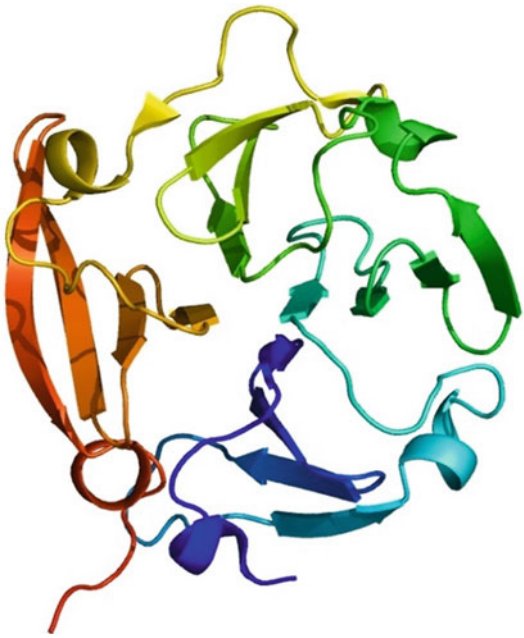
been identified as a novel predictor of cardiovascular risk in patients with coronary artery disease (Blankenberg et al. 2003) and stroke (Montaner et al. 2003).

Clinical Markers

High blood pressure increases the risk of coronary artery disease. Coronary artery disease leads to narrowing of the arteries over time. The narrowed artery limits or blocks the flow of blood to the heart muscle; the hardened surface of the artery can also encourage the formation of small blood clots. People with high blood pressure are more likely to develop coronary artery disease because high blood pressure puts added force against the artery walls. Over time, this extra pressure can damage the arteries. These injured arteries are more likely to become narrowed and hardened by fatty deposits. Damaged arteries cannot deliver enough oxygen to other parts of the body. For this reason, high blood pressure can harm the brain and kidneys. High blood pressure also increases the risk for stroke, congestive heart failure, kidney disease, and blindness systolic and diastolic blood pressure (Mancia et al. 2014).

Carotid intima-media thickness measurements are being applied widely as a measure of atherosclerosis in studies on determinants of presence and progression of atherosclerosis and in studies on atherosclerosis as determinant of cardiovascular disease. Carotid intima-media thickness is a measure of early atherosclerosis and vascular remodeling that can be assessed quickly, non-invasively, and cheaply with high-resolution ultrasound. Carotid intima-media thickness has been shown to be related to cardiovascular risk factors, prevalent cardiovascular disease, and atherosclerosis in the peripheral, coronary, and femoral arteries. Recently, evidence became available indicating that an increased C-IMT is a strong predictor of coronary heart

Fig. 5 MMP-9 structure



disease and stroke (Society of Atherosclerosis Imaging and Prevention Developed in collaboration with the International Atherosclerosis Society 2011). Therefore, it has been suggested that measurements of C-IMT may be used to identify high-risk subjects.

Metabolic Markers

Some metabolic parameters are involved in cardiovascular risk, they include:

- *Clinical markers*: total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, postprandial glucose, lipoprotein(a) [Lp(a)], homocysteine, apolipoprotein A-1 (Apo A-I), Apo B, uric acid

It is widely accepted that cardiovascular disease is associated with elevated levels of LDL cholesterol, total cholesterol, and triglycerides and low levels of HDL cholesterol. Apolipoprotein A-1 is the major protein of HDL lipoprotein, while Apo B is the major protein of very low, low and intermediate-density lipoproteins. Because of their associations with the respective lipoproteins, Apo A-1 is inversely and Apo B is positively associated with cardiovascular risk (Criqui et al. 1993). Apolipoprotein A-1 is a main initiator and "driver of the reverse cholesterol transport." Apolipoprotein A-1 can also manifest anti-oxidant and anti-inflammatory effects, and it can stimulate both endothelial production of nitric oxide (NO) as

well as release of prostacyclin from the endothelium (Hoang et al. 2007). Apo B has been shown to be superior to LDL cholesterol in predicting the risk of vascular events and the progression of vascular disease in a series of prospective epidemiological studies. As previously said, Apo B is a component of all atherogenic or potentially atherogenic particles, including very-low-density lipoprotein, intermediate-density lipoprotein, LDL, and Lp(a), each particle contains one molecule of Apo B. Therefore, Apo B provides a direct measure of the number of atherogenic lipoprotein particles in the circulation. An accumulating body of data indicates that the Apo B/Apo A-1 ratio is a powerful marker of risk for future cardiovascular disease (Walldius and Jungner 2006).

While LDL performs the necessary function of delivering lipids for cellular use, its higher lipid to protein ratio means that these particles are subject to becoming trapped within the walls of arteries. The mechanism by which LDL attaches itself to damaged intima in the arterial tree is complex. LDL carries not only Apo B but other proteins including lipoprotein lipase. Lipoprotein lipase (LPL) is synthesized in tissue parenchymal cells and then translocated to functional binding sites on the luminal surfaces of endothelial cells. Proteoglycans are also important players in the attachment of LDL particles to the vascular wall and act as a docking mechanism for the LDL particles. Once the LDL particle has been attached to the endothelial surface, further changes must occur before the cholesterol-rich particle becomes part of the atherosclerotic plaque. In an artery wall, an LDL molecule becomes vulnerable to oxidation by free radical attack. Macrophages have a high affinity for oxidized LDL (oxLDL). Oxidized LDL is recognized by a macrophage scavenger receptor inducing these cells to take up lipid by attempted phagocytosis. Macrophages are unable to process oxLDL in this manner causing lipids to accumulate on the immune cells transforming them into a type of cellular debris called foam cells. The foam cell is, perhaps, the hallmark of the atherosclerotic lesion (Sierra-Johnson et al. 2009). The generation of these cells is associated with imbalance of cholesterol influx, esterification, and efflux (Fig. 6). The disruption of immune function can result in chronic inflammation, and the ongoing availability of excess lipids coupled with aggregation of immune cells in a region may result in accumulation of foam cells locally creating the hallmark of the disease (Tomkin and Owens 2012).

Lipoprotein(a) is a variant of LDL that is covalently linked to Apo B. Concentrations change widely through different populations and more than 90 % of this variant is determined by inherited DNA sequence variation (Kathiresan 2009). Evidence that Lp(a) is a cause rather than a consequence of coronary artery disease has been strengthened by a report by Robert Clarke et al. (2009), who found that two Lp(a) single-nucleotide polymorphisms explain 36 % of the variation in Lp (a) in their population. The mechanism whereby Lp(a) may be particularly atherogenic is through its binding and transportation of phospholipids (Enkhmaa et al. 2011). However, due to the particle homology with plasminogen, a proenzyme related to fibrinolysis (Koschinsky 2005), it is suggested that the particle also plays a part in thrombosis. Combining a proatherogenic factor with an antifibrinolytic factor, makes Lp(a) an interesting candidate for a link between plaque and stenosis and a

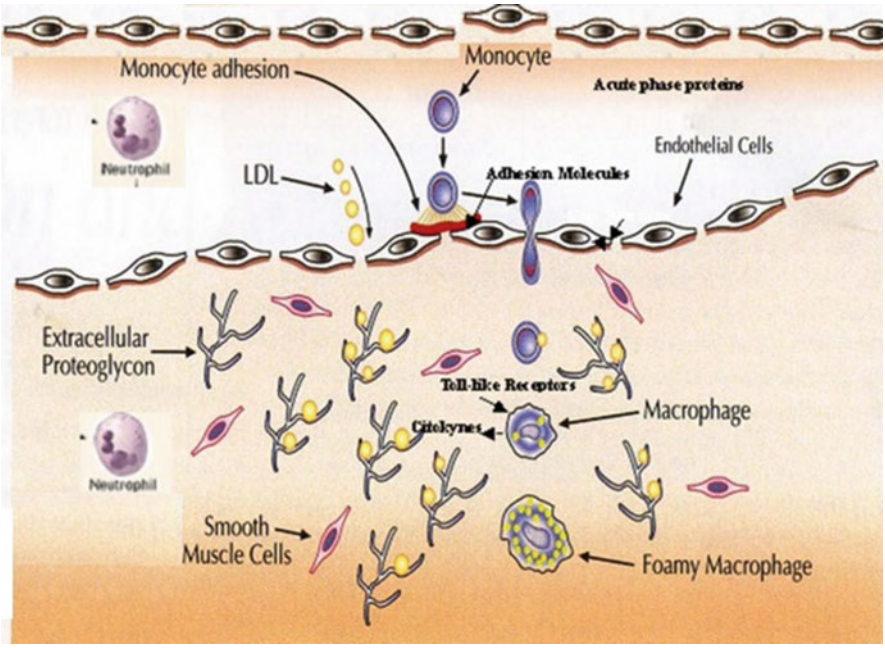


Fig. 6 Mechanism of atherosclerosis. (Adapted from Licastro et al. 2005).

likely risk factor for thrombotic events, including atherosclerotic occlusion (Berglund and Ramakrishnan 2004). More recently, Lp(a) has been shown to interfere with annexin A5 binding to the procoagulant phosphatidyl serine. Annexin 5 is involved in anticoagulation on the endothelial surface and thus this is another mechanism whereby Lp(a) might promote thrombosis (Fu et al. 2010).

Postprandial glucose is a major risk factor for both the microvascular and macrovascular complications in patients with type 2 diabetes (Aryangat and Gerich 2010). Acute hyperglycemia has been linked to endothelial dysfunction, in particular postprandial fluctuations, and, in addition to absolute increases in glycemia, contribute to oxidative stress and endothelial dysfunction. Oral glucose tolerance test (OGTT) is the best experimental model to evaluate the metabolic answer of the single patient to a meal. Previous published studies reported that after OGTT there was a significant increase in biomarkers of systemic low-grade inflammation and endothelial dysfunction such as Hs-CRP, IL-6, TNF-α, sICAM-1, sVCAM-1, and sE-selectin (Derosa et al. 2010b, c). Oxidative stress caused by acute postprandial glycaemia spikes can contribute to macrovascular damage through oxidation of low-density lipoprotein, exacerbation of endothelial dysfunction, and other proatherogenic mechanisms. Hyperglycemia enhances the secretion of endothelin-1, a vasoconstrictor, in vitro and decreases NO production in the aorta of diabetic rat and coronary microvessels in human. Moreover, postprandial glycaemia induces glycation of protein which forms cross linked protein termed advanced glycation end

products (AGE), with consequent synthesis and release of cytokines, vasoadhesion molecule, endothelin-1, and tissue factor.

Hyperhomocysteinaemia is associated with an increased risk of cardiovascular diseases that can lead to stroke or heart attack (Nygård et al. 1997). Homocysteine causes endothelial cell dysfunction and induces apoptotic cell death in cell types relevant to atherothrombotic disease, including endothelial cells (Hossain et al. 2003) and smooth muscle cells. The highly reactive thiol group of homocysteine is readily oxidized to form reactive oxygen species (Starkebaum and Harlan 1986), suggesting that homocysteine induces cell injury/dysfunction through a mechanism involving auto-oxidation and oxidative damage. Moreover, cell culture studies have demonstrated that homocysteine induces the production of several proinflammatory cytokines.

Finally, an increase of uric acid (hyperuricaemia) plays a role in cardiovascular risk, and for this reason, it should be considered. Patients affected by chronic hyperuricaemia and urate deposition have an increased risk of developing coronary disease (Abbott et al. 1988). The Health Professionals Follow-up study showed that hyperuricaemic and gouty arthritic patients had a higher mortality risk for cardiovascular diseases than those patients who had coronary disease (Choi and Curhan 2007). In addition to this, recent evidence has supported the concept that hyperuricaemia itself can be a significant and independent cardiovascular risk factor, not only for cardiovascular and cerebrovascular diseases but also for renal failure, hypertension, and type 2 diabetes (Grassi et al. 2013).

Potential Applications to Prognosis, Other Diseases or Conditions

The discovery and early identification of biomarkers linked to cardiovascular disease has added a new layer to our knowledge of pathophysiology and is opening up new avenues for prognostication, diagnosis, and therapy. Biomarkers are an important tool to better identify high-risk patients, in order to diagnose disease conditions promptly and accurately, and to effectively prognosticate and treat patients. This can help to reduce the costs linked to cardiovascular disease and reduce days of hospitalization.

Summary Points

- This chapter focuses on biomarkers relevant to cardiovascular diseases.
- Biomarkers include measurable indicators of some biological state and are useful to diagnose or follow-up a specific condition or risk factor.
- Biomarkers relevant to cardiovascular diseases include markers of insulin resistance (HOMA index, adiponectin, retinol binding protein-4), inflammation (tumor necrosis factor-α, high-sensitivity C-reactive protein, myeloperoxidase, interleukin-6, paraoxonase-1), and endothelial damage (adhesion molecules, metalloproteinases).

- Recently, accurate, efficient, reliable, and sensitive analytical methods for the determination of these biomarkers have been developed.
- Early identification of biomarkers linked to cardiovascular disease can be effective in identifying high-risk patients and early treating them in order to reduce cardiovascular disease.

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Use of Multiple Biomarkers to Estimate Cardiovascular Drug Efficacy: Advantage of a PRE Score

2

Paul A. Smink and Hiddo L. J. Heerspink

Contents

Abstract

In cardiovascular disease, drugs are targeted toward normalizing a single risk factor, the on-target effect. The ultimate goal of drug treatment is to provide long-term cardiovascular organ protection. In recent years, several trials have shown that drugs with promising effects on the on-target risk factor failed to improve long-term cardiovascular protection. One explanation for these failures is that a drug does not only affect the risk factor to which it is targeted but also other

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_47

parameters, off-target effects, which may also affect long-term cardiovascular outcomes. The drug effect on these off-target effects may be as large or even larger than the on-target effect. The off-target drug effects may consequently have an important impact on the drug effect on cardiovascular outcomes.

This chapter provides an overview of on-target and off-target effects of drugs used in cardiovascular risk management. Keynote in this chapter is that ignoring off-target effects of a drug may lead to severe misinterpretations about the long-term cardiovascular protective effect, with major consequences for society and individual patients. To solve this problem, all effects of a drug should be incorporated into a risk algorithm to obtain a more accurate estimation of the drug effect on long-term cardiovascular outcome.

Keywords

Biomarkers • Drug effects • Cardiovascular complications • Clinical trials • Drug development

Abbreviations

ACEi	Angiotensin-converting-enzyme inhibitor
ADVANCE	Action in diabetes and vascular disease: preterax and diamicron MR-controlled evaluation
ALTITUDE	Aliskiren trial in type 2 diabetes using cardiorenal end points
ARB	Angiotensin receptor blocker
DRI	Direct renin inhibitor
HDL-C	High-density lipoprotein cholesterol
hs-CRP	High-sensitivity C-reactive protein
IDNT	Irbesartan diabetic nephropathy trial
LDL-C	Low-density lipoprotein cholesterol
NT-proBNP	N-terminal pro-braintype natriuretic peptide
PRE score	Parameter response efficacy score
RAAS	Renin-angiotensin-aldosterone system
RENAAL	Reduction of Endpoints in NIDDM with the Angiotensin II Antagonist Losartan study
UKPDS	UK Prospective Diabetes Study

Key Facts

- Cardiovascular disease is a major global health concern, accounting for four million deaths in Europe each year.
- Cardiovascular disease is usually characterized by systemic atherosclerosis, which is a process that involves endothelial plaque formation eventually in micro- and macro-vascular disease.
- In order to slow down this progress and to prevent occurrence of fatal or nonfatal cardiovascular complications, many patients require multiple drug treatments.

- In the past 20 years, several treatments have been proven effective in reducing cardiovascular events, and this has resulted in a steady decrease in the incidence of cardiovascular disease.
- However, with an increasing prevalence of obesity in both developed and developing countries, combined with high salt intake, consumption of fatty foods, a sedentary lifestyle, and ongoing smoking habits, it is questionable whether the achievements in reducing cardiovascular morbidity and mortality can be sustained on the long term.
- New innovative treatment strategies are recommended to mitigate the burden of cardiovascular disease.

Definitions

Biomarker A laboratory measurement that serves as an indicator of a physiological or pathophysiological process or as a response to treatment which affects such a process.

Cardiovascular risk factor A biomarker which has a direct causal relationship with cardiovascular disease.

Dose finding The process to find the dose of the drug with optimal efficacy and safety.

Hard outcome clinical trial Drug study in which the actual long-term effect of a drug (e.g., preventing myocardial infarction or stroke) is established on clinically meaningful outcomes.

On-target effect Drug effect on the cardiovascular risk factor to which the drug is targeted to.

Off-target effect Drug effects on parameters beyond the on-target effect.

PRE score Algorithm which involves short-term drug-induced changes in on-target and many off-target cardiovascular risk markers and integrates these short-term changes into a score which denotes the chances of long-term cardiovascular risk change (reduction or increase).

Renin-angiotensin-aldosterone system (RAAS) Hormonal system which regulates sodium and water excretion and blood pressure.

Risk engine Algorithms such as Framingham, UKPDS, and ADVANCE which are developed to provide individual long-term (i.e., 5 or 10 years) risk estimations to develop cardiovascular complications based on the individual presence of cardiovascular risk factors including age, gender, smoking habits, and diabetes.

Introduction

Cardiovascular protective drugs are targeted toward an effect on a single cardiovascular risk marker. For example, a cardiovascular protective drug is targeted toward lowering blood pressure (for antihypertensive drugs), toward changing lipid profiles (with either low-density lipoprotein (LDL)-lowering or high-density lipoprotein (HDL)-increasing drugs), or toward lowering HbA1c (for oral glucose-lowering drugs). However, targeting a drug toward a single cardiovascular risk factor is not a goal at itself but a mean to determine whether a drug is organ protective. In current drug development processes, a drug is considered to be organ protective if the drug has a substantial effect on the cardiovascular risk factor which it is targeted to within a short time period (e.g., 3 or 6 months), the on-target effect. The assumption made is that the on-target effect will translate into long-term cardiovascular protection and that the drug does not have any other effect on risk factors, off-target effects, that may influence long-term outcome as well. This implies that the on-target drug effect serves as a surrogate/proxy to estimate the long-term cardiovascular efficacy. If positive, this estimation justifies the conduct of a large hard outcome clinical trial in which the actual long-term protective effect (e.g., preventing myocardial infarction or stroke) is established in a time period of about 4 years. Safety of a drug is typically ascertained by monitoring the drug effect on a regular set of "safety" parameters, which are usually less rigorously determined compared to the drug effect on the on-target parameter. This approach of estimating long-term cardiovascular protection has resulted in registration and authorization of several drugs which are currently used in clinical practice (Cohen 2010, 856–865; Zhao et al. 2009, 315–325).

Off-Target Effects

The fact that a drug has a substantial effect on the cardiovascular risk factor which it is targeted to does not necessarily imply that the drug delivers the expected long-term cardiovascular protection. Recent cases have illustrated this notion. Sibutramine was launched in 1999 as a weight-lowering drug which should improve cardiovascular outcome. However, a few years after registration, excessive cases of hypertension and tachycardia were reported leading to cardiovascular events among sibutramine users. Therefore, in 2010, European authorities decided to suspend marketing authorization (James et al. 2010, 905–917). Rosiglitazone received marketing authorization in 2000 as an HbA1c-lowering drug, which was supposed to improve prognosis in patients with type 2 diabetes. However, after its introduction to the market, meta-analyses revealed that rosiglitazone increased risk of myocardial infarction and heart failure, despite its consistent HbA1c-lowering effect. The increased heart failure incidence could be attributed to renal tubular sodium retention leading to excessive extracellular fluid retention and weight gain, which fueled discussions about the safety of rosiglitazone. Eventually, marketing authorization of rosiglitazone was suspended in 2010 (Blind et al. 2011, 213–218; Nissen and Wolski 2010, 1191–1201). The development program of torcetrapib, a cholesteryl

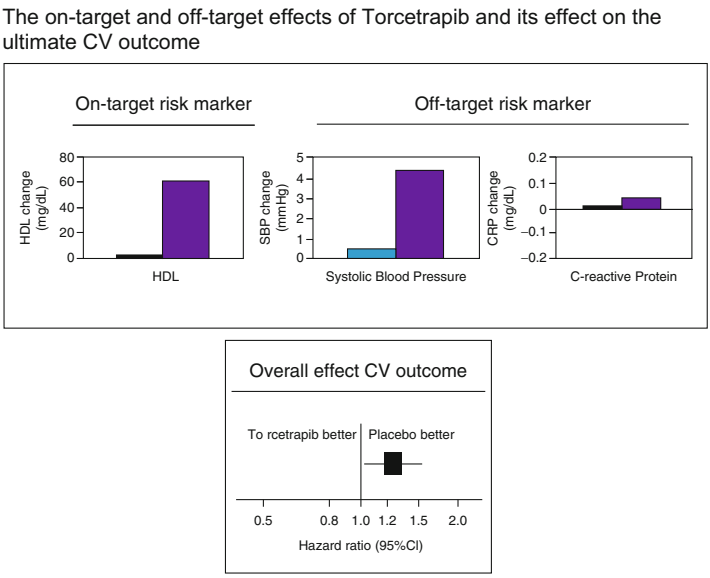

Fig. 1 Effect of the cholesteryl esterase protein inhibitor torcetrapib on on-target (HDL-C) and off-target risk markers and cardiovascular outcome (Data derived from (Barter et al. 2007, 2109–2122). *CI* confidence interval, *HDL-C* high-density lipoprotein cholesterol)

ester transfer protein-inhibiting and high-density lipoprotein cholesterol (HDL-C)-raising drug, was prematurely terminated, because a hard outcome trial showed no improvement in cardiovascular outcome despite increases in HDL cholesterol (Barter et al. 2007, 2109–2122). Additional investigations revealed that this unexpected finding was attributable to a rise in blood pressure as a consequence of increased mineralocorticoid activity, which possibly counteracted the beneficial effect of the drug on HDL-C (Fig. 1; Hu et al. 2009, 2211–2219; Sofat et al. 2010, 52–62). A more recent example of a drug in this drug class is dalcetrapib. In a large outcome trial, the drug indeed increased HDL cholesterol, but it did not lead to significant reductions in cardiovascular risk compared to placebo. Dalcetrapib increased systolic blood pressure and C-reactive protein. These effects may have increased cardiovascular risk and may have blunted the degree of cardiovascular protection with dalcetrapib (Schwartz et al. 2012).

These cases of drug failure in late-stage drug development teach us that targeting a drug to a single biomarker may lead to serious misinterpretations of the actual long-term drug effect, with major consequences for society and individual patients. In all these cases, the drug had effects on other parameters than the target risk parameter alone. Currently, these so-called off-target effects are considered as side effects, which implies less rigorous measurement and reporting. Estimating the drug effect on cardiovascular morbidity or mortality by only taking the on-target drug effect into account may be problematic. Firstly, the off-target effects may also influence long-term cardiovascular protection as shown in Fig. 2.

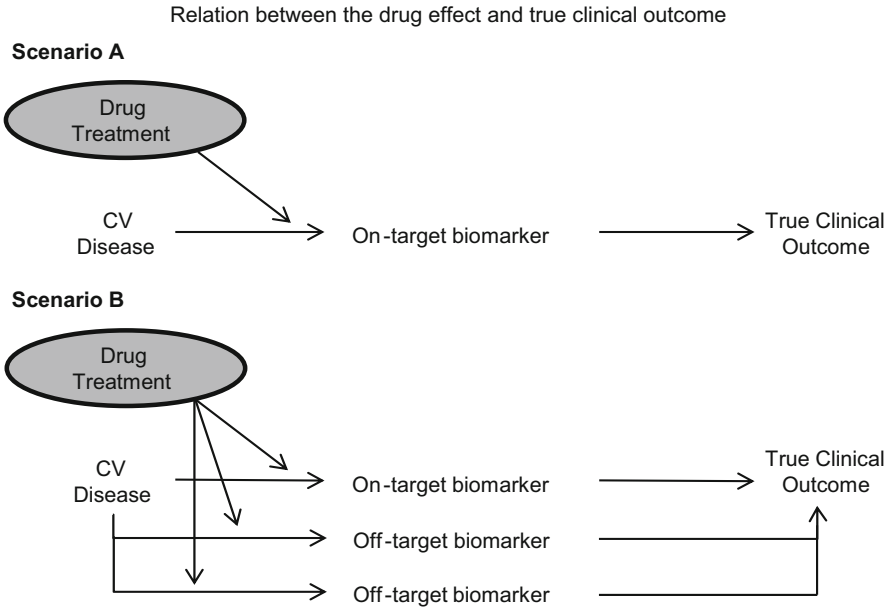

Fig. 2 Representation of on-target and off-target biomarkers determining true clinical outcome. In scenario A, the drug is assumed to affect the on-target biomarker alone, which completely explains the drug effect on true clinical outcome. In scenario B, the drug is assumed to affect off-target biomarkers as well, which also contribute to the ultimate drug effect on the true clinical outcome. *CV* Cardiovascular

Secondly, the response in the on-target and off-target parameters may be different between individuals. For example, angiotensin receptor blockers are registered for blood pressure lowering. However, these drugs also decrease albuminuria and hemoglobin and increase serum potassium. It appears that individual patients show a wide variability in responses in these multiple parameters, indicating that the response in the off-target parameters cannot be estimated from the response in blood pressure. As drug-induced changes in blood pressure, albuminuria, hemoglobin, and serum potassium are all associated with cardiovascular risk, combining the drug effect on multiple parameters may be a more rational approach to estimate drug effects on hard cardiovascular outcomes instead of using the drug effect on a single parameter.

A New Proposal: The PRE Score

How can a drug effect on multiple biomarkers be integrated into a composite drug response which acquires a more accurate estimation of the long-term drug effect? First, insights must be obtained what cardiovascular risk factors are affected during

drug treatment on short term and to what extent these changes in risk factors influence long-term cardiovascular outcome. Then, these on-target and off-target effects should be integrated into a composite response score which relates the change in multiple parameters to the long-term cardiovascular outcome. Such composite risk scores consisting of multiple risk markers already exist for predicting cardiovascular risk of individual patients. A well-known example is the Framingham risk score. Similar multiple parameter scores should be developed for predicting a drug effect on cardiovascular outcomes as well. One such score, the multiple risk parameter response efficacy (PRE) score has been developed. This score involves short-term drug-induced changes in on-target and many off-target cardiovascular risk markers and integrates these short-term changes into a score which denotes the chances of long-term cardiovascular risk change (reduction or increase) (Fig. 3).

The PRE score was tested and validated in already completedrandomized controlled trials in patients with type 2 diabetes and nephropathy either assigned to losartan or placebo (RENAAL trial) or irbesartan or placebo (IDNT trial). Both losartan and irbesartan are registered as antihypertensive drugs. As shown in Fig. 4, both losartan and irbesartan appeared to have *nine* short-term off-target effects beyond blood pressure lowering. Changes in any single risk marker failed to predict the ultimate drug effect on renal/CV outcome in the trials. However, the PRE score, integrating all available risk markers, accurately predicted the ultimate drug effect on long-term cardiovascular outcome (Fig. 5; Smink et al. 2014b, 208–215). Subsequently, the PRE score was validated by predicting the long-term effect of a direct renin inhibitor (DRI) aliskiren on top of conventional RAAS-blocking agents (ACEi or ARB). It was shown that aliskiren, in contrast to what was expected based on estimations of the on-target drug effect, would only moderately improve renal and cardiovascular outcomes (Smink et al. 2014a, 434–441). The early termination of the ALTITUDE trial confirmed the lack of effect of aliskiren on top of conventional RAAS blockade (Parving et al. 2012, 2204–2213).

The PRE Score and Dose Finding

Another aspect to consider is that off-target and on-target effects of a drug may be dose dependent. Proper dose selection is required to provide information on the dose beyond which no additional cardiovascular protection will be established or even harm as a result of off-target effects setting off the on-target effect. Choosing the right dose for the hard outcome trial is a crucial decision in the design of trials, and a wrong dose selection may result into failure of the trial. An example of the wrong dose selection is the avosentan drug development. In a phase 2b study with the endothelin antagonist avosentan, dose-dependent reductions in albuminuria were shown with an apparent maximum albuminuria-lowering effect of avosentan at doses of 10 mg/day. Higher doses up to 50 mg/day were tested but they had no additional effect on albuminuria, but higher avosentan doses dose-dependently increased body weight as a consequence of fluid retention. For the phase 3 trial

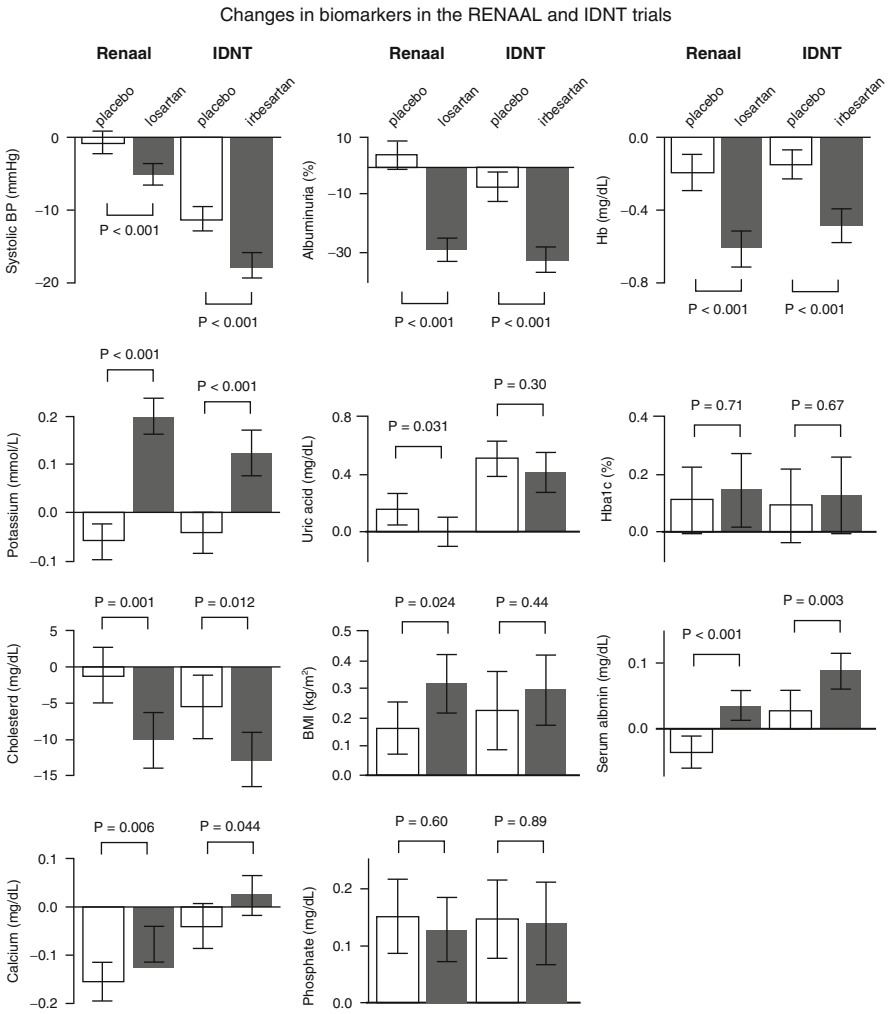

Fig. 3 Change in biomarkers after 6 months placebo or ARB treatment in the RENAAL and IDNT trials. *ARB* angiotensin receptor blocker, *IDNT* Irbesartan Diabetic Nephropathy Trial, *RENAAL* Reduction of Endpoints in NIDDM with the Angiotensin II Antagonist Losartan trial (With permission from *Clinical Pharmacology and Therapeutics*)

with avosentan in patients with diabetic nephropathy, it was decided to use a 25 mg/day and 50 m/g dose (Wenzel et al. 2009, 655–664). Unfortunately, the phase 3 trial was early terminated due to excesses of heart failure and mortality due to fluid retention in the avosentan groups (Mann et al. 2010, 527–535). Of course, it is always easy to judge dose selection in hindsight, but this example illustrates the importance of involving off-target drug effects in the dose selection during drug development. Currently, dose selection is based on changes in a single on-target risk

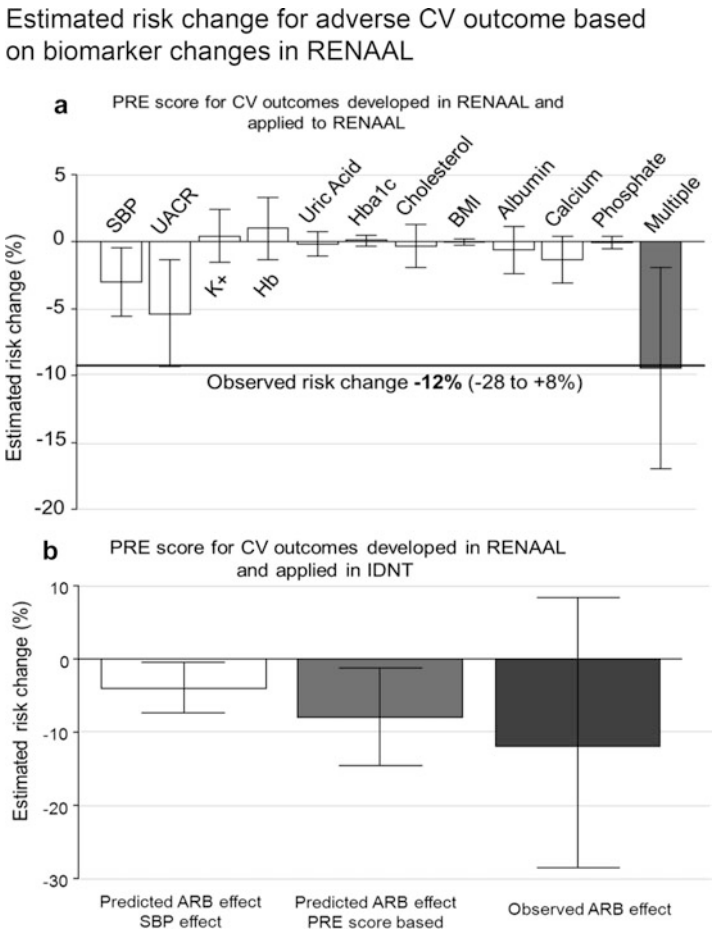

Fig. 4 Observed and predicted long-term relative renal and cardiovascular risk change (%) based on single and multiple PRE scores. The actual observed treatment effect is indicated by the solid line. The predicted treatment effect based on single and multiple PRE scores are shown by the vertical bars. The PRE score was developed in the RENAAL trial and applied to the baseline and month 6 values of the placebo and losartan treatment arm of the RENAAL trial. (**a**) Validation of the PRE score in the IDNT trial. The PRE score is developed in the RENAAL trial and applied to the baseline and month 6 measurements of the irbesartan and placebo arm of the IDNT trial. (**b**) *IDNT* Irbesartan Diabetic Nephropathy Trial, *PRE* parameter response efficacy, *RENAAL* Reduction of Endpoints in NIDDM with the Angiotensin II Antagonist Losartan trial (With permission from *Clinical Pharmacology and Therapeutics*)

factor. However, given that drugs have multiple effects on cardiovascular risk factors, it appears more appropriate to select the optimal dose based on changes in multiple cardiovascular risk factors. Application of the PRE score is one strategy to select optimal drug doses during drug development based on multiple cardiovascular risk factors.

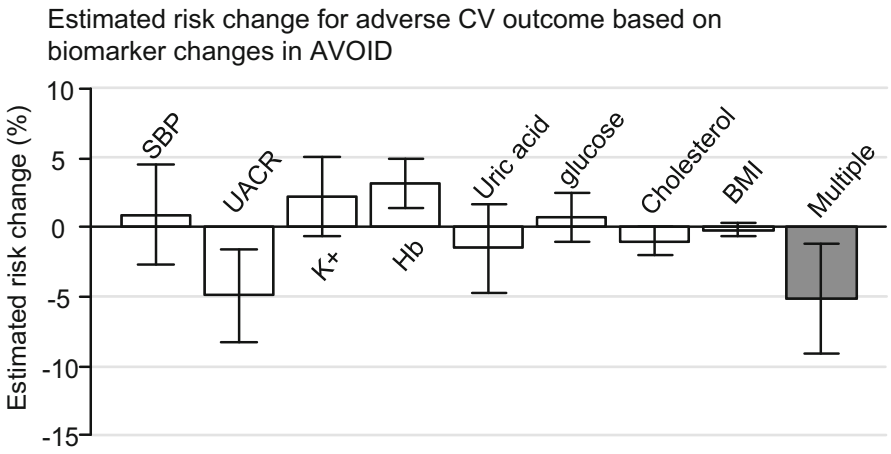

Fig. 5 Estimated relative risk change for the cardiovascular end point based on single and multiple PRE scores. *CV* cardiovascular, *PRE* parameter response efficacy (With permission from *European Journal of Preventive Cardiology*)

Potential Applications to Prognosis, Other Disease, and Conditions

What is the novelty of the PRE score? As described above, the PRE score may provide an accurate estimation about (lack of) long-term organ protection that follows from treatment. This estimation is based on a score in which multiple treatment-induced biomarker effects are incorporated. This is not a novel concept and already in use by current cardiovascular risk engines such as Framingham, UKPDS, and ADVANCE. However, estimations provided by these risk engines are based entirely on traditional cardiovascular risk factors including blood pressure, cholesterol, hba1c, etc. (Stevens et al. 2001, 671–679; Kengne et al. 2011; Wilson et al. 1998, 1837–1847). Studies have shown that individual cardiovascular risk profiles cannot be determined by traditional cardiovascular risk factors alone but should also include novel biomarkers as albuminuria, hs-CRP, NT-proBNP, etc. (Folsom et al. 2006, 1368–1373). PRE score-based estimations incorporate drug-induced changes in novel biomarkers. This has the potential to lead to more accurate drug efficacy estimations.

What are the implications of the PRE score? PRE score-based predictions of long-term organ protection are based on the short-term effect on multiple biomarkers, and therefore fewer patients will be unnecessarily exposed to ineffective or even harmful drugs. Furthermore, the PRE score can be used to perform dose selection, which may be beneficial for clinical trial conduction. Finally, the PRE score may contribute in optimizing drug treatment in daily clinical practice. An integrated score including the on-target and off-target effects may offer the physician and the patient a more

reliable tool to estimate and evaluate the overall prescribed drug effect on long-term outcomes. Changes in off-target effects may preclude adjusting or stopping treatment, despite absence of a substantial effect on the on-target parameter. This could be relevant for the patient-clinician dialogue.

Conclusion

Currently, drugs in cardiovascular disease are targeted toward a single biomarker. The ultimate goal is to reduce cardiovascular morbidity and mortality. Several cases have taught us that the single biomarker approach may lead to serious misinterpretations about the long-term cardiovascular protective effect of the drug. The PRE score, which incorporates multiple short-term biomarker effects of a drug, provides a more accurate insight of the long-term cardiovascular protective effect.

Summary Points

- Drugs in cardiovascular disease are targeted toward normalizing a single cardiovascular risk factor, the on-target effect, whereas the ultimate goal is to provide long-term cardiovascular protection.
- Several cases have shown that cardiovascular drugs were effective in normalizing cardiovascular risk factors but fail to provide long-term protection.
- These unexpected findings could be attributable to the drug effect on other parameters, the off-target effects.
- Ignoring the off-target effects of a drug may lead to severe misinterpretations of the drug effect on long-term cardiovascular outcome with major consequences for society and individual patients.
- To obtain a more accurate estimation of the drug effect on long-term cardiovascular outcome, all effects of a drug should be incorporated into a risk algorithm, the PRE score.

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Cardiovascular Disease Biomarkers in Clinical Use and Their Modulation by Functional Foods

3

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Contents

Abstract

Biomarkers are conventionally defined as "biological molecules that represent health and disease states." Type 2 diabetes, dyslipidemia, and hypertension are strong risk factors for cardiovascular disease (CVD), a leading cause of morbidity and mortality worldwide. Consequently, biomarkers reflecting blood glucose,

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_37

conventional lipid profiles, blood pressure, and inflammation (e.g., C-reactive protein (CRP)), that are routinely used in clinical practice, are effective in predicting CVD. Functional foods, particularly berries, cocoa, and tea have been shown to lower blood glucose and improve insulin sensitivity in some studies, and in most they have beneficial effects on conventional lipids. Soy as a functional food in adults has been associated with lowering of total and LDL cholesterol levels. Emerging evidence supports the role of fruits and vegetables, cocoa, and tea in decreasing CRP, though we did not observe such effects following supplementation of berries and tea in adults with "prediabetes." Consistent observations support the antihypertensive effects of berries, cocoa, and tea in adults with "prediabetes" or advanced CVD. Dietary bioactive compounds, especially polyphenols, have been shown to mediate biological mechanisms that lead to the modulation of clinical biomarkers. Thus, selected functional foods that are commonly consumed in the daily diet hold promise for CVD and can lower levels of biomarkers associated with disease progression.

Keywords

Biomarkers • Berries • Cocoa • Tea • LDL cholesterol • C-reactive protein • Blood pressure

Abbreviations

ALT	Alanine aminotransferase
apoB	Apolipoprotein B
BMI	Body mass index
BP	Blood pressure
CAD	Coronary artery disease
CHD	Coronary heart disease
CJ	Cranberry juice
CJC	Cranberry juice cocktail
CRP	C-reactive protein
CT	Computed tomography
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DC	Dark chocolate
EGCG	Epigallocatechin gallate
F&V	Fruits and vegetables
FBF	Forearm blood flow
FBG	Fasting blood glucose
FDA	Food and drug administration (US)
FDB	Freeze-dried blueberries
FDS	Freeze-dried strawberries
FFWC	Flavanol-free white chocolate

FMD	Flow-mediated dilatation
FRDC	Flavanol-rich dark chocolate
GT	Green tea
GTE	Green tea extracts
HDL-C	High-density lipoprotein cholesterol
HOMA-IR	Homeostatic model assessment of insulin resistance
HTN	Hypertension
IR	Insulin resistance
ISP	Isolated soy protein
LDL-C	Low-density lipoprotein cholesterol
NAFLD	Non-alcoholic fatty liver disease
NCEP	National Cholesterol Education Program
NEFA	Non-esterified fatty acids
NMR	Nuclear magnetic resonance
PJ	Pomegranate juice
QUICKI	Quantitative insulin sensitivity check index
SAA	Serum amyloid A
SBP	Systolic blood pressure
T2D	Type 2 diabetes
TC	Total cholesterol
TG	Triglycerides
TLC	Therapeutic lifestyle change
VCAM-1	Vascular cell adhesion molecule 1
VLDL-C	Very low-density lipoprotein cholesterol

Key Facts Related to Blood Glucose and Lipid/Lipoproteins and Their Modulation by Functional Foods

- Blood glucose values greater than 126 mg/dL (fasting state) and HbA1c greater than 6.5 % are indicative of diabetes.
- Blood lipids, especially LDL cholesterol greater than 120 mg/dL, represent independent risk factor for CVD.
- Berries, especially blueberries and strawberries, lower total and LDL cholesterol and improve insulin sensitivity in adults with CVD risk factors.
- Green tea lowers glucose and LDL cholesterol in adults with the metabolic syndrome, the prediabetic state.
- Whole soy foods lower serum lipids in adults with total cholesterol greater than 200 mg/dL and/or LDL cholesterol greater than 120 mg/dL.
- Polyphenols and other bioactive compounds in functional foods can reduce absorption and metabolism of dietary carbohydrates and lipids.
- Polyphenols and other bioactive compounds in functional foods can increase LDL receptor activity and reduce hepatic cholesterol synthesis.

Key Facts Regarding Inflammatory Biomarkers Modulated by Functional Foods

- Biomarkers of inflammation such as CRP, fibrinogen, and adhesion molecules are indicators of progression of CVD.
- Values of CRP greater than 3 mg/L are indicative of increased CVD risk.
- Fruits and vegetables, cocoa, and tea lower CRP in some reported studies.
- Our research did not find any effects of green tea and berry supplementation on CRP.
- A combination of functional foods may be more effective in lowering inflammation than a single agent.

Key Facts Regarding Modulation of Blood Pressure by Functional Foods

- Hypertension, defined as systolic blood pressure greater than 140 mmHg and/or diastolic blood pressure greater than 90 mmHg, is an independent risk factor for CVD.
- Metabolic syndrome identifies systolic blood pressure greater than 130 mmHg and diastolic blood pressure greater than 85 mmHg as increasing risks of CVD.
- Cocoa, berries, and tea lower blood pressure in many reported studies.
- Our research found blueberries to lower blood pressure in adults with the metabolic syndrome, the prediabetic state.
- Polyphenols and other bioactive compounds in functional foods can improve dilation of the blood vessels.

Definitions

Biomarkers Biological molecules that represent health and disease states; example, blood glucose.

Cardiovascular disease (CVD) A class of diseases that involve the heart or blood vessels; common CVDs include: ischemic heart disease (IHD), stroke, hypertensive heart disease, rheumatic heart disease (RHD), aortic aneurysms, cardiomyopathy, atrial fibrillation, congenital heart disease, endocarditis, and peripheral artery disease (PAD), among others.

C-reactive protein Acute phase protein synthesized by the liver; commonly measured biomarker of inflammation.

Functional foods Foods that provide health benefits beyond basic nutrition; example, green tea.

Hypertension Elevated systolic and diastolic blood pressure; independent risk factor of CVD.

Inflammation Natural immune response; chronic inflammation linked to various diseases, such as CVD and cancer.

LDL cholesterol One of the five major groups of lipoproteins and a common carrier of blood cholesterol typically measured in health and disease states; elevated levels associated with increased risk of CVD.

Metabolic syndrome A "prediabetic state" defined as elevated glucose, elevated blood pressure, elevated triglycerides, reduced HDL cholesterol, and abdominal obesity; any three of these five components confer a diagnosis of the metabolic syndrome.

Polyphenols Major category of plant-based bioactive compounds in foods and beverages shown to confer protection against chronic diseases including cardiovascular disease; exert antioxidant and vasodilator actions among others; example, catechins in green tea

Type 2 diabetes Caused by a progressive insulin secretory defect on the background of insulin resistance; diagnosis involves elevated fasting or 2-h postchallenge blood glucose and/or glycated hemoglobin (HbA1c)

Introduction

Cardiovascular disease (CVD), including coronary heart disease (CHD) and stroke, is the leading cause of mortality worldwide, and thus a target for intensive lifestyle and dietary intervention, pharmacological intervention, or both (Go et al. 2013; Roth et al. 2015). Biomarkers, defined as biological molecules that can detect and monitor clinical and subclinical disease burden and response to treatments, have been routinely used in the screening and management of CVD (Jensen et al. 2014). The well-known Framingham Heart Study established the importance of traditional risk factors, such as diabetes, smoking, elevated total cholesterol and reduced high-density lipoprotein (HDL) cholesterol levels, hypertension, and overweight/obesity, as predictors of CVD (D'Agostino et al. 2008). Based on these observations, several algorithms involving CVD biomarkers have been developed to predict an individual's absolute risk of CVD (Jensen et al. 2014; Wenger 2014). The use of biomarkers of glycemia, lipidemia, inflammation (e.g., C-reactive protein (CRP)), and vascular function, such as blood pressure and arterial elasticity, has become an integral part of the clinical care in CVD. These biomarkers have been extensively studied in response to dietary exposures of nutrients and dietary bioactive compounds.

Functional foods and nutraceuticals have gained popularity in the scientific community because of their health benefits that extend beyond basic nutrition, and

several have been shown to exert protective effects against CVD (Crowe and Francis 2013). Berries, cocoa, soy, and tea deserve special attention among the commonly consumed foods and beverages for their cardio-protective effects. The aim of this chapter is to understand the role of these functional foods in modulating CVD biomarkers, based on evidence from clinical studies, including controlled feeding studies reported by our group.

Biomarkers of Blood Glucose and Lipids Are Modulated by Berries, Cocoa, and Tea

Blood glucose and lipid/lipoprotein profiles have been established as biomarkers of type 2 diabetes (T2D) and atherosclerotic CVD in hallmark epidemiological studies and have been extensively used in routine clinical care to identify high-risk populations (Jensen et al. 2014; Wenger 2014). Elevated fasting glucose ($\geq$126 mg/dL) and HbA1c ($\geq$6.5 %) are key diagnostic criteria for diabetes mellitus and are also used to identify the prediabetic state, the metabolic syndrome (fasting plasma glucose 100–125 mg/dL; HbA1c 5.7–6.4 %) (American Diabetes Association 2014). Elevated blood lipids, particularly high LDL ($\geq$120 mg/dL), is an independent risk factor for CVD and a biomarker that is commonly targeted by intervention studies aimed at lowering lipids and subsequent CVD risk (Wenger 2014). Dyslipidemia as characterized by the metabolic syndrome (triglycerides $\geq$150 mg/dL; HDL cholesterol $<$50 mg/dL for women and $<$40 mg/dL for men) is also a risk factor for atherosclerotic CVD, a common vascular complication of diabetes. Based on current understanding of the pathophysiology of insulin resistance, diabetes, and atherosclerotic CVD, multiple pharmacological and non-pharmacological interventions have been developed with the aim of improving blood glucose and lipids, thus lowering risks of vascular complications (Fig. 1).

Berries, cocoa, and tea have demonstrated significant effects in lowering CVD biomarkers, and most of their effects have been attributed to bioactivity of polyphenolic flavonoids, in combination with other compounds, such as phytosterols and fiber in these foods and beverages. In Tables 1 and 2, we present a summary of selected clinical studies that report significant findings on the effects of berries, cocoa, and tea in modulating blood glucose and lipids in participants with one or more CVD risk factors. The baseline ranges of average values of blood glucose and conventional lipids reported in these studies are summarized as follows: glucose (80–155 mg/dL), HbA1c (5.5–7.5 %), total cholesterol (138–239 mg/dL), LDL cholesterol (90–156 mg/dL), HDL cholesterol (38–55 mg/dL), and triglycerides (97–195 mg/dL). Among the 20 studies summarized in Tables 1 and 2, berries, cocoa, or tea supplementation was demonstrated to decrease insulin resistance (improve insulin sensitivity) and/or decrease fasting blood glucose in only eight (Grassi et al. 2008; Stull et al. 2010; Almoosawi et al. 2010; Udani et al. 2011; Sarriá et al. 2014; Nagao et al. 2009; Liu et al. 2014; Mozaffari-Khosravi et al. 2014). On the other hand, berries, cocoa, or tea supplementation was shown to favorably modulate one or more biomarkers of conventional lipid profiles in most of

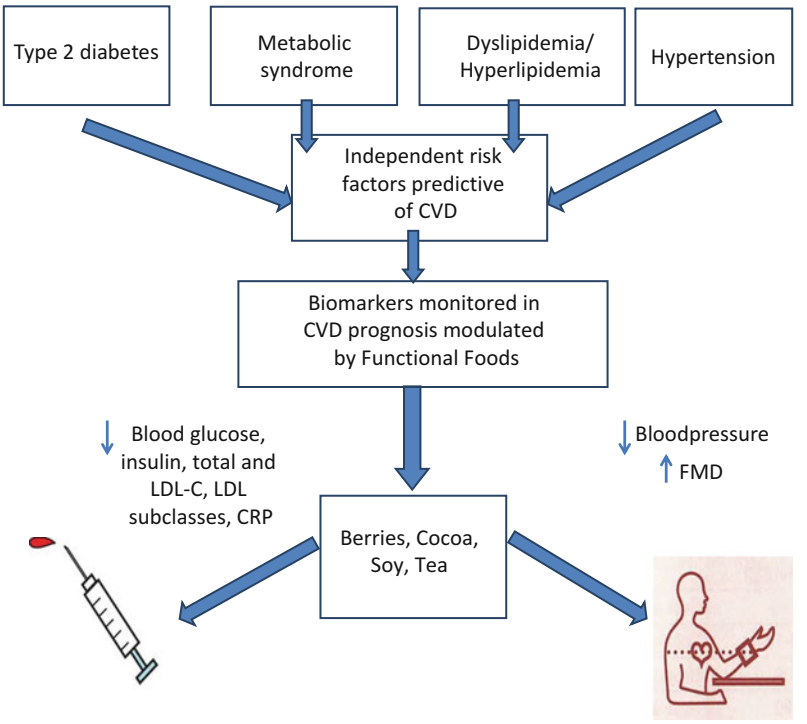

Fig. 1 Clinical biomarkers of CVD and functional foods

the studies reported (Grassi et al. 2008; Udani et al. 2011; Sarriá et al. 2014; Nagao et al. 2009; Liu et al. 2014; Mozaffari-Khosravi et al. 2014; Ruel et al. 2006; Balzer et al. 2008; Mellor et al. 2010; Zunino et al. 2012; Basu et al. 2014, 2010a; Maron et al. 2003; Unno et al. 2005; Nagao et al. 2007; Hsu et al. 2008). We conducted a randomized dose-response feeding trial examining the effects of low (25 g/day) and high (50 g/day) doses of freeze-dried strawberries on glucose and lipid profiles in obese participants with elevated lipids. Our results showed significant decreases in total and LDL cholesterol, as well as nuclear magnetic resonance (NMR)-derived small LDL particle concentrations in the high-dose strawberry group when compared to the controls. No differences were noted in serum glucose, triglycerides, or HDL cholesterol (Basu et al. 2014). In a similar study (Zunino et al. 2012) freeze-dried strawberries were also shown to decrease total cholesterol and increase NMR-derived LDL particle size in obese adults. Another of our studies of people with metabolic syndrome following green tea beverage supplementation (four cups/day) showed trends toward lower LDL cholesterol and higher HDL cholesterol when compared to the unsupplemented controls (Basu et al. 2010). All of these are small studies, but suggest that further research is indicated on the role of berries and green tea in modulating blood glucose and lipids across the spectrum of CVD risks.

Table 1 Modulation of blood glucose and lipids by dietary berries and cocoa in clinical studies of participants with CVD risk factors

Author, year	Study design and duration	Subject characteristics	Intervention	Effects on blood lipids	Effects on blood HbA1c, glucose, insulin, and IR
Ruel et al. (2006)	Placebo phase followed by three successive phases of increasing doses of CJC; 16 weeks	Sedentary men with elevated waist circumference and LDL-C, $n = 30$, mean age = 51 year, BMI = 27.8 kg/m^2	Low-calorie CJC (125 ml, 250 ml, or 500 ml/day)	Increased plasma HDL-C after 250 ml CJC/day; no effects in total-, LDL-, and VLDL-C	Not reported
Grassi et al. (2008)	Randomized crossover; 15 days	Hypertensive men ($n = 11$) and women ($n = 8$) with impaired glucose intolerance, mean age = 45 year; BMI = 26.5 kg/m^2	FRDC or FFWC (100 g/day)	Decreased total and LDL-C after FRDC; no effects on HDL-C and TG	Decreased IR and increased QUICKI; increased insulin sensitivity and beta-cell function after FRDC
Balzer et al. (2008)	Randomized, double-blind, placebo-controlled; 30 days	Adults with T2D, $n = 41$, mean age = 64 year, BMI = 31.6 kg/m^2	Flavanol-rich cocoa (321 mg flavanols) or nutrient-matched control (25 mg flavanols); 3 times daily	Decreased LDL-C only after flavanol-rich cocoa	No significant effects
Stull et al. (2010)	Randomized, double-blind, and placebo-controlled; 6 weeks	Obese, nondiabetic, and insulin-resistant adults, $n = 32$, mean age = 51.5 year, BMI = 37.4 kg/m^2	Blueberry bioactives (22.5 g) or placebo; twice daily	No significant effects	Increased insulin sensitivity after blueberry treatment
Mellor et al. (2010)	Randomized, double-blind, placebo-controlled crossover; 8 weeks	Adults with T2D, $n = 12$, age = 42–71 year, BMI = not reported, body weight at study end = 89 kg	Chocolate with high or low polyphenol content (45 g/day)	Increased HDL-C; decreased total: HDL-C after high-polyphenol chocolate	No significant effects
Almoosawi et al. (2010)	Randomized, single-blind, crossover; 2 weeks	Overweight and obese adults, $n = 14$, age = 21–50 year, BMI = 27.7 kg/m^2	DC (500 mg or 1,000 mg polyphenols); 20 g/day	No significant effects	Decreased FBG with both doses of DC

Udani et al. (2011)	Open label pilot clinical study; 1 month	Overweight adults, $n = 10$, mean age = 28 year, BMI = 27.4 kg/m^2	Acai pulp (100 g) twice daily	Decreased total-C after acai treatment	Decreased FBG and plasma fasting insulin after acai treatment
Zunino et al. (2012)	Randomized, double-blind, placebo-controlled, crossover; 7 weeks	Healthy obese adults, $n = 20$, mean age = 31 year, BMI = 34.0 kg/m^2	Strawberry powder (equivalent to 160 g each serving frozen strawberries) or strawberry-flavored control; twice daily for 3 weeks each	Decreased total cholesterol, NMR-derived small HDL-C particles, increased LDL particle size after strawberry treatment	No significant effects on glucose
Sarria et al. (2014)	Randomized, controlled crossover; 4 weeks	Moderately hypercholesterolemic adults, $n = 20$, mean age = 31 year, BMI = 24.3 kg/m^2	Cocoa product in milk (15 g each serving) twice daily or milk (control)	Increased HDL-C after cocoa treatment; no effect on total- and LDL-C and TG	Decreased FBG after cocoa treatment
Basu et al. (2014)	Randomized, dose-response controlled; 12 weeks	Adults with abdominal obesity and elevated serum lipids, $n = 60$, mean age = 49 year, BMI = 36 kg/m^2	FDS (low dose, 25 g, or high dose, 50 g) or fiber- and calorie-matched control beverages daily	Decreases in total- and LDL-C and NMR-derived small LDL particles in high vs. low dose FDS and vs. high dose controls; no effects on HDL-C and TG	No significant effects on glucose and insulin resistance

The above table is a summary of human intervention studies examining the effects of different forms of dietary berry fruits and juices and cocoa products on levels of blood glucose, conventional lipids, and lipoprotein subclasses in participants with CVD risk factors

Table 2 Modulation of blood glucose and lipids by tea in clinical studies of participants with CVD risk factors

Author, year	Study design and duration	Subject characteristics	Intervention	Effects on blood lipids	Effects on blood HbA1c, glucose, insulin, and IR
Maron et al. (2003)	Randomized, double-blind, placebo-controlled, parallel trial; 12 weeks	Adults with mild to moderate hypercholesterolemia on a low-fat diet, $n = 240$, mean age = 55 year, BMI = 24.2 kg/m^2	Theaflavin-enriched GTE (375 mg polyphenols) or placebo	Decreased total and LDL-C after GTE treatment	Not reported
Unno et al. (2005)	Randomized, triple-crossover postprandial study; 6 h	Adult men with borderline to mild hypertriacylglycerolemia, $n = 9$, mean age = 46 year; BMI = 26.8 kg/m^2	Green tea catechins (10 mg, control), moderate (224 mg), or high (674 mg) dose with a light meal (bread with 20 g butter)	Attenuated postprandial rise of plasma triacylglycerols after green tea treatment; no effects in TC and NEFA	Not reported
Nagao et al. (2007)	Randomized, double-blind, controlled trial; 12 weeks	Adults with abdominal obesity, $n = 240$, mean age = 42 year, BMI = 26.5 kg/m^2	Green tea catechins (583 mg) or control (96 mg) daily	Decreased LDL-C after green tea treatment	No significant effects
Mackenzie et al. (2007)	Randomized, double-blind, placebo-controlled trial; 3 months	Adults with T2D not taking insulin, $n = 49$, mean age = 65 year, BMI = 33.3 kg/m^2	Extracts of green tea and black tea (0, 375 mg or 750 mg polyphenols/day)	Not reported	No significant effects
Hsu et al. (2008)	Randomized, double-blind, placebo-controlled trial; 12 weeks	Obese women, $n = 78$, mean age = 44 year, BMI = 30.9 kg/m^2	GTE (400 mg) three times daily or placebo	Decreased LDL-C and TG, increased HDL-C after GTE treatment	No significant effects

Nagao et al. (2009)	Randomized, double-blind, controlled trial; 12 weeks plus 4-week follow-up	Adults with T2D not taking insulin, $n = 43$, mean age = 64 year, BMI = 24.8 kg/m^2	GT beverage [96 mg (control) or 583 mg catechins/340 mL/]	Decreased total cholesterol and FFA after GT treatment	Decreased HbA1c and increased insulin
Basu et al. (2010b)	Randomized, single-blind, controlled trial; 8 weeks	Adults with the metabolic syndrome, $n = 35$, mean age = 42 year, BMI = 36.3 kg/m^2	Green tea beverage (4cups), green tea extracts (2 capsules), or water (control); EGCG content similar in both green tea groups	Decreasing trend in LDL-C and LDL/HDL and increasing trend in HDL-C after GT treatment	No significant effects
Vieira Senger et al. (2012)	Randomized, controlled trial; 60 days	Adults with the metabolic syndrome, $n = 45$, age $\geq$60 year, BMI = 30.5 kg/m^2	GT (3 cups/day) or no green tea	No significant effects	No significant effects
Liu et al. (2014)	Randomized, double-blind, placebo-controlled trial; 16 weeks	Adults with T2D, $n = 77$, mean age = 54 year, BMI = 26.3 kg/m^2	GTE (500 mg polyphenols/day) three times daily or placebo	Decreased TG, increased HDL-C after GTE treatment	Decreased insulin and HOMA-IR
Mozaffari-Khosravi et al. (2014)	Randomized clinical trial; 4 weeks	Adults with T2D, $n = 94$, mean age = 52 year, BMI = 28.2 kg/m^2	GT or sour tea (150 mL) three times daily	Increased HDL-C after GT and sour tea	Decreased insulin in GT and increased insulin in sour tea group; decreased HOMA-IR in GT vs. sour tea

The above table is a summary of human intervention studies examining the effects of different forms of tea as beverage or extracts on levels of blood glucose, conventional lipids, and fatty acids in participants with CVD risk factors

Many mechanistic studies explain the role of berries, cocoa, and tea bioactive compounds in the management of blood glucose and lipids. The hypoglycemic effects of polyphenols are mainly attributed to their ability to reduce intestinal absorption of dietary carbohydrates, modulation of the enzymes involved in glucose metabolism, improvement of β-cell function and insulin action, stimulation of insulin secretion, and the antioxidative and anti-inflammatory properties of these compounds (McDougall et al. 2005; Munir et al. 2013; Hanhineva et al. 2010). In case of blood lipid/lipoprotein profiles, polyphenols have been shown to decrease lipid absorption from the intestine and formation of micelles, cause inhibition of cholesterol absorption from brush-border membranes, inhibition of cholesterol synthesis, and decreased hepatic secretion of apolipoprotein B (apoB)-100 (Chen et al. 2014; Bladé et al. 2010). Thus, future clinical studies must define the role of berries, cocoa, and tea in modulating blood glucose and lipids in the context of variations in habitual diet, metabolic phenotypes, optimal dosing, and effects of food processing on bioactivities of constituent compounds.

Modulation of Lipids and Lipoproteins by Soy

As shown in many clinical studies and in systematic meta-analyses over the last two decades, soy products, such as soy proteins, soy phytoestrogens, and soy nuts can reduce serum lipids and lipoproteins (Anderson et al. 1995; Zhan and Ho 2005; Anderson and Bush 2011). These beneficial findings have been adopted for the development of preventive strategies against CVD. The US Food and Drug Administration (FDA) approved the health claim that "25 g of soy protein a day, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease" (Food and Drug Administration 1999). Table 3 summarizes the effects of soy as a functional food on lipids and lipoprotein profiles in participants with elevated lipids. The baseline range of average values of conventional lipids reported in these studies are summarized as follows: total cholesterol (208–270 mg/dL), LDL cholesterol (136–186 mg/dL), HDL cholesterol (45–62 mg/dL), and triglycerides (112–192 mg/dL). Out of these eight studies, five showed a significant decrease in LDL cholesterol following soy supplementation per se or in combination with a cholesterol-lowering diet (Crouse et al. 1999; Wangen et al. 2001; Tonstad et al. 2002; Blum et al. 2003; Welty et al. 2007). Apolipoprotein B (apoB) was shown to decrease in only one of these studies (Welty et al. 2007), while HDL cholesterol and triglycerides were mostly unaffected. Based on these studies and the meta-analyses, it appears that soy exerts cholesterol-lowering effects largely in participants with elevated total and LDL cholesterol values. Furthermore, clinical responses to soy has also been shown to be modulated by equol production, a product of intestinal bacterial metabolism of soy isoflavone daidzein (Hodis et al. 2011), though not all reported studies comment on the role of equol in modulating the effects of soy on lipid profiles. The mechanisms responsible for the effects of soy on serum lipoproteins continue to being explored, but have been mainly attributed to the role of soy isoflavones in modulating LDL receptor activity

Table 3 Modulation of lipids and lipoproteins by soy in clinical studies of participants with CVD risk factors

Author, year	Study design and duration	Subject characteristics	Intervention	Effects on blood lipids and lipoproteins
Crouse et al. (1999)	Randomized, double-blind, parallel trial; 9 weeks	Moderately hypercholesterolemic adults on a NCEP Step I diet, $n = 156$, mean age = 52 year, BMI = 26 kg/m^2	ISP (25 g with 3 mg, 27 mg, 37 mg, or 62 mg isoflavones/day) or 25 g casein	Decreased total and LDL-C after 62 mg isoflavones from ISP; decreased total and LDL-C after 37 mg isoflavones from ISP daily in high LDL-C group
Wangen et al. (2001)	Randomized, crossover trial; 93 days (three phases)	Normocholesterolemic and mildly hypercholesterolemic postmenopausal women, $n = 18$, mean age = 57 year, BMI = 25.2 kg/m^2	ISP [7 mg (control), 65 mg, or 132 mg isoflavones/day)	Decreased LDL-C after 132 mg isoflavones from ISP; decreased LDL: HDL-C after 65 mg and 132 mg isoflavones from ISP
Tonstad et al. (2002)	Randomized, parallel trial; 16 weeks	Adults with LDL-C $\geq$4 mmol/L, $n = 130$, mean age = 52 year, BMI = 24.9 kg/m^2	ISP (30 g or 50 g/day) and cotyledon fiber or matched casein and cellulose fiber beverage on a lipid-lowering diet	Decreased LDL-C after 30 g and 50 g ISP
Blum et al. (2003)	Randomized, double-blind, placebo-controlled, crossover trial; 6 weeks	Postmenopausal women with hypercholesterolemia, $n = 24$, mean age = 55 year, BMI not reported	Soy protein (25 g/day) or placebo (milk protein)	Decreased total and LDL-C after 25 g soy protein as well as placebo
Welty et al. (2007)	Randomized, controlled, crossover trial; 8 weeks	Healthy normo and hypertensive postmenopausal women, $n = 60$, mean age = 56 year, BMI = 26.7 kg/m^2	Soy nuts (one-half cup/day; 25 g soy protein) or TLC diet	Decreased LDL-C and apoB in hypertensive women after soy nut intake; no effects in normotensive women

(*continued*)

Table 3 (continued)

Author, year	Study design and duration	Subject characteristics	Intervention	Effects on blood lipids and lipoproteins
Hodis et al. (2011)	Randomized, double-blind, placebo-controlled trial; 2.7 years	Postmenopausal women, $n = 350$, mean age = 61 year, BMI = 25.6 kg/m^2	ISP (25 g with 91 mg aglycon isoflavone equivalents) or placebo	Increased HDL-C after 25 g ISP
Liu et al. (2012)	Randomized, double-blind, placebo-controlled trial; 6 months	Postmenopausal women with prediabetes or early untreated T2D, $n = 180$, mean age = 56 year, BMI = 24.5 kg/m^2	15 g milk protein+100 mg isoflavones/day, or 15 g soy protein+100 mg isoflavones/day, or 15 g milk protein (placebo)	No significant effects
Acharjee et al. (2015)	Randomized, controlled, crossover trial; 8 weeks	Healthy postmenopausal women with or without the metabolic syndrome, n = 60, mean age = 54 year, BMI = 28.2 kg/m^2	Soy nuts (one-half cup/25 g soy protein and 101 mg aglycone isoflavones/day) or TLC diet	Decreased TG only after soy nut intake in women with the metabolic syndrome

The above table is a summary of human intervention studies examining the effects of different forms of soy products on blood levels of conventional lipids and lipoproteins in participants with CVD risk factors

and hepatic cholesterol synthesis (Anderson et al. 1995; Zhan and Ho 2005). Thus, whole soy foods rather than isolated soy components, in combination with a healthy diet, in individuals with elevated total and LDL cholesterol may have cholesterol-lowering effects. Further studies are needed to assess these lipid-lowering effects of soy on CVD complications.

Biomarkers of Inflammation Modulated by Flavonoid-Containing Foods and Beverages

A significant amount of information has been gathered in the last few years on the role of functional foods, especially those containing polyphenols, in modulating biomarkers of inflammation. Inflammation has been proposed as the major pathologic mechanism underlying the development and progression of atherosclerotic CVD (Willerson and Ridker 2004). Many surrogate biomarkers of inflammation have been identified and positively correlated with the initiation and progression of endothelial damage leading to atherosclerosis. Some of these key inflammatory

biomarkers are the following: adhesion molecules, C-reactive protein (CRP), cytokines, fibrinogen, and serum amyloid A (SAA). Table 4 summarizes selected intervention studies on the role of polyphenol-containing foods and beverages in modulating biomarkers of inflammation. Among these eight studies, five reported a decrease in CRP, a routinely measured serum biomarker of inflammation in clinical practice (Dong et al. 2011; Kolehmainen et al. 2012; Stote et al. 2012; Moazen et al. 2013; Macready et al. 2014). However, in our own work, we did not observe any significant differences in inflammatory markers, including CRP and adhesion molecules, following blueberry, strawberry, or green tea intervention (Basu et al. 2014, 2010a, b, 2011). It appears that the baseline levels of these biomarkers, study duration, as well as use of single vs. combined functional foods are important factors that underpin differences observed in target inflammatory molecules.

Large-scale prospective cohort studies have shown significant utility of CRP and fibrinogen in predicting cardiovascular events. In these studies, it was demonstrated that following an initial screening with conventional risk factors alone, the additional assessment of CRP or fibrinogen in people at intermediate risk for a cardiovascular event could help prevent one additional event over a period of 10 years for every 400–500 people so screened (Kaptoge et al. 2012). Though CRP levels vary in different populations, a CRP value >3 mg/L has been shown to be independently associated with a 60 % excess risk in incident CHD compared with levels <1 mg/L after adjustment for all Framingham risk variables (Yousuf et al. 2013). Thus, as shown in Table 4, the role of cocoa, fruits and vegetables, soy, and tea in reducing CRP and/or fibrinogen means that their anti-inflammatory functions deserve further evaluation in larger studies of populations with or without CVD complications.

Modulation of Blood Pressure and Vascular Compliance by Berries, Cocoa, and Tea

Hypertension is the strongest risk factor for CVD and is clinically defined as systolic blood pressure (SBP) ≥140 mmHg and/or diastolic blood pressure (DBP) ≥90 mmHg (James et al. 2014). The metabolic syndrome or the "prehypertensive" state identifies cut points of above normal systolic (≥130 mmHg) and diastolic (≥85 mmHg) blood pressure that have also been shown to be associated with increased risk of CVD (Go et al. 2013). Thus, blood pressure, arterial elasticity, and related measures of vascular compliance are common biomarkers of CVD and can be modified by lifestyle modifications including diet and physical activity. The role of polyphenol-containing foods in the management of blood pressure and vascular dysfunction is inherent in the established guidelines for prevention of hypertension, especially those emphasizing the consumption of fruits and vegetables which are naturally high in polyphenols and other cardio-protective nutrients (Kokubo 2014). Table 5 summarizes the role of polyphenol-containing functional foods and beverages in modulating blood pressure and/or markers of endothelial function and arterial compliance. Among these nine studies, five showed decreases in systolic and/or diastolic blood pressure following interventions with berries, tea,

Table 4 Modulation of C-reactive protein and other markers of inflammation by flavonoid-containing functional foods in clinical studies of participants with CVD risk factors

Author, year	Study design and duration	Subject characteristics	Intervention	Effects on CRP	Effects on other biomarkers of inflammation
Basu et al. (2011)	Randomized, controlled trial; 8 weeks	Adults with the metabolic syndrome, $n = 35$, mean age = 42.5 year, BMI = 36.1 kg/m^2	Green tea beverage (4cups), green tea extracts (2 capsules), or water (control); EGCG content similar in both green tea groups	No significant effects	Decrease in SAA
Dong et al. (2011)	Meta-analysis of 14 randomized controlled trials	Postmenopausal women	Soy foods with isoflavones or isoflavone extracts as treatment vs. controls	Decrease in CRP in women with baseline CRP >2.2 mg/L after isoflavone treatment	Not reported
Kolehmainen et al. (2012)	Randomized, controlled trial; 16 weeks	Overweight adults with features of the metabolic syndrome, $n = 27$, mean age = 52 year, BMI = 32.0 kg/m^2	Bilberries (400 g) or control diet daily	Decreasing trends in CRP after bilberry treatment	Decreased inflammation score
Stote et al. (2012)	Randomized crossover trial with increasing doses of cocoa and/or tea flavanols; 5 days each	Adults with obesity and insulin resistance, $n = 19$, mean age = 46 year, BMI = 36.8 kg/m^2	Cocoa (30, 180, 400, 900 mg) or green tea (2.4 g), daily	Decreased CRP in the highest vs. lowest cocoa dose	Decreased fibrinogen after green tea intervention

Moazen et al. (2013)	Randomized, controlled trial; 8 weeks	Adults with T2D, $n = 36$, mean age = 52 year, BMI = 28.0 kg/m^2	FDS beverage (50 g/day) or macronutrient-matched placebo powder	Decreased CRP after strawberry intervention	Not reported
Sakata et al. (2013)	Randomized, double-blind controlled trial; 12 weeks	Patients with NAFLD, $n = 17$, mean age = 51 year, BMI = 29 kg/m^2	Green tea at high (1,080 mg), or low catechin doses (200 mg), or a placebo	Not reported	Decreased serum ALT and improved liver-to-spleen CT attenuation ratio after green tea treatment
Macready et al. (2014)	Randomized, dose-dependent, controlled trial; 6 weeks	Adults with CVD risk factors, $n = 174$, mean age = 51 year, BMI = 27.6 kg/m^2	High-flavonoid F&V; low-flavonoid F&V and habitual F&V	Decreased CRP in the high-flavonoid F&V group	Decreased E-selectin and VCAM
Asgary et al. (2014)	Randomized, controlled trial; 2 weeks	Adults with HTN, $n = 21$, mean age = 53 year, BMI = 27.4 kg/m^2	Pomegranate juice (150 ml) or similar amount of water daily	No significant effects	Decreased VCAM, increased E-selectin

The above table is a summary of human intervention studies examining the effects of different flavonoid-containing functional foods on blood levels of inflammation in participants with CVD risk factors

or pomegranate juice (Brown et al. 2009; Basu et al. 2010a; Hodgson et al. 2013; Mozaffari-Khosravi et al. 2013; Asgary et al. 2014), while others using cocoa or berry supplementation showed no effect on blood pressure but an improvement in flow-mediated dilation (FMD) (Balzer et al. 2008; Dohadwala et al. 2011). The reported studies are mostly in participants on antihypertensive medications. The baseline range of average values of systolic and diastolic blood pressure reported in these studies are 123–136 mmHg and 73–87 mmHg, respectively. We reported blood pressure-lowering effects of freeze-dried blueberries (50 g/day) in obese adults with the metabolic syndrome (Basu et al. 2010b). However, no such effects on blood pressure or markers of endothelial function were noted in other studies reported by our group involving freeze-dried strawberries (Basu et al. 2014) or green tea (Basu et al. 2010) in obese participants with one or more CVD risk factors. Thus, the effects of functional foods may be modulated by their specific makeup of polyphenols and other nutrients and their interaction with vascular function across the disease continuum.

The literature describes several synergistic mechanisms that account for the antihypertensive effect of polyphenols, acting through different molecular targets and improving endothelium-dependent vasodilation. Inflammation, endothelial dysfunction, and oxidation are apparently interrelated mechanisms that play a substantial role in the pathogenesis of hypertension and are mitigated or reversed by functional foods rich in polyphenols (Huang et al. 2013). However, limited clinical data are available and further research is needed to identify the optimal dosing of these foods and beverages for sustained effects on blood pressure in populations at risk of CVD.

Potential Applications to Prognosis, Other Diseases, or Conditions

CVD is often a lifelong disease that begins with the evolution of risk factors that in turn contribute to the development of subclinical atherosclerosis. The onset of CVD itself worsens the prognosis, with great risk of recurrent event, morbidity, and mortality. Biomarkers of blood glucose, lipids, and blood pressure that are commonly used in clinical practice play a critical role in defining the long-term prognosis of diabetes and atherosclerotic CVD. Blood glucose and HbA1c are significant predictors of CVD complications (Cederberg et al. 2010), and thus their modulation by functional foods is a subject of emerging interest in the secondary prevention of these conditions. LDL cholesterol lowering is an important goal: a 2–3 mmol/L reduction is associated with a 40–50 % reduction of CVD "events" (Baigent et al. 2010). In the case of blood pressure control, studies have reported an 18 % risk reduction of stroke mortality with as little as a 5 mmHg reduction in systolic blood pressure (Lackland et al. 2014). Though the magnitude of effects of functional foods is typically less than that of drug interventions, these foods and beverages as part of a long-term daily diet hold promise in the modulation of biomarkers associated with CVD. Thus, future research must identify their effectiveness in high risk

Table 5 Modulation of blood pressure and vascular compliance by selected functional foods and beverages in clinical studies of participants with CVD risk factors

Author, year	Study design and duration	Subject characteristics	Intervention	Effects on systolic and diastolic blood pressure	Effects on markers of endothelial function/ arterial compliance
Balzer et al. (2008)	Randomized, double-blind, placebo-controlled; 30 days	Adults with T2D, $n = 41$, mean age = 64 year, BMI = 31.6 kg/m^2	Flavanol-rich cocoa (321 mg flavanols) or nutrient-matched control (25 mg flavanols); 3 times daily	No significant effects	Increased FMD with cocoa intervention
Brown et al. (2009)	Randomized, double-blind, placebo-controlled trial; 8 weeks	Overweight/obese male adults, $n = 88$, mean age = 51 year, BMI = 31.1 kg/m^2	EGCG capsule (400 mg) twice daily or placebo	Decreased diastolic BP; decreasing trends in systolic BP after EGCG treatment	Not reported
Basu et al. (2010b)	Randomized, single-blind, controlled trial; 8 weeks	Adults with the metabolic syndrome, $n = 35$, mean age = 42 year, BMI = 36.3 kg/m^2	Green tea beverage (4cups), green tea extracts (2 capsules), or water (control); EGCG content similar in both green tea groups	No significant effects	Not reported
Basu et al. (2010a)	Randomized, controlled trial; 8 weeks	Adults with the metabolic syndrome, $n = 48$, mean age = 50 year, BMI = 37.8 kg/m^2	FDB (25 g with 480 mL water) twice daily or control (no blueberries)	Decreased systolic and diastolic BP after blueberry treatment	Not reported
Dohadwala et al. (2011)	Acute uncontrolled pilot study; 4 h and randomized, double-blind, placebo-controlled crossover trial; 4 weeks	Adults with CAD, $n = 59$, mean age = 62 year, BMI = 29.5 kg/m^2	Double-strength CJ (480 mL; 835 mg polyphenols/day) or placebo	No significant effects	Increased FMD after acute CJ dose; decreased carotid-femoral pulse wave velocity after CJ treatment for 4 weeks

(*continued*)

Table 5 (continued)

Author, year	Study design and duration	Subject characteristics	Intervention	Effects on systolic and diastolic blood pressure	Effects on markers of endothelial function/ arterial compliance
Droste et al. (2013)	Randomized, prospective, unblinded trial; 20 weeks	Adults with carotid atherosclerosis, $n = 108$, mean age = 64 year, BMI = 27.6 kg/m^2	Red wine (100 mL for women, 200 mL for men/day) or no alcohol in the setting of a Mediterranean diet and physical activity	No significant effects	Not reported
Hodgson et al. (2013)	Randomized, double-blind, controlled trial; 6 months	Adults with systolic BP between 115 and 150 mmHg and diastolic BP <100 mmHg, $n = 111$, mean age = 57 year, BMI = 25.2 kg/m^2	Powdered black tea solids (3 cups/day with 429 mg polyphenols) or flavonoid-free beverage	Decreased systolic and diastolic BP during nighttime after black tea treatment	Not reported
Mozaffari-Khosravi et al. (2013)	Randomized, clinical trial; 4 weeks	Adults with mild hypertension and T2D, $n = 100$, mean age = 52 year, BMI = 28.2 kg/m^2	GT or sour tea (150 mL) three times daily	Decreased systolic and diastolic BP after GT and sour tea treatment	Not reported
Asgary et al. (2014)	Randomized, controlled trial; 2 weeks	Adults with HTN, $n = 21$, mean age = 53 year, BMI = 27.4 kg/m^2	Pomegranate juice (150 ml) or similar amount of water daily	Decreased systolic and diastolic BP after PJ treatment	Decreased VCAM-1, increased E-selectin after PJ treatment; no effects on FMD

The above table is a summary of human intervention studies examining the effects of different flavonoid-containing functional foods on blood pressure and vascular compliance in participants with CVD risk factors

populations and associations with other novel biomarkers, such as those related to genomics, epigenomics, proteomics, and metabolomics in the prognosis and management of CVD.

Summary Points

- Biomarkers of blood glucose, conventional lipids, CRP, and blood pressure in clinical practice play an important role in prognosis and management of CVD.
- Berries, cocoa, and tea lower blood glucose and lipids (conventional and NMR-derived subclasses) in participants with elevated CVD risk factors.
- Whole soy foods can lower total and LDL cholesterol, most effectively in people with elevated values in conventional lipid profiles.
- Polyphenol-containing fruits and vegetables, berries, and teas lower CRP in a few studies but effect on other inflammatory biomarkers are not well-defined.
- Berries, cocoa, and tea lower systolic and diastolic blood pressure but effect on soluble markers of endothelial function and arterial elasticity are not well-defined.

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Biomarker-Guided Therapy for Chronic Heart Failure

4

Alexander E. Berezin

Contents

Abstract

The interest in guided therapy for acutely decompensated and chronic heart failure using several biological markers, which vary according to the pathogenesis of cardiac failure, has been steadily increasing. The circulating levels of brain natriuretic peptide (BNP) and N-terminal prohormone of BNP (NT-pro-BNP) are routinely used in clinical practice to stratify the risk of patients with symptomatic chronic heart failure. This chapter discusses the goal of lowering concentrations of these markers and their continued suppression in the follow-up period as part of the current therapeutic approach to chronic heart failure. Although a recent

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_1

European Society of Cardiology (ESC) guideline did not recommend biomarker-guided therapy based on BNP/NT-pro-BNP in the management of chronic heart failure patients, the American Heart Association/American College of Cardiology (AHA/ACC) clinical practice guidelines for heart failure have issued a class I and A-level of evidence for BNP/NT-pro-BNP, citing them as powerful tools to supplement clinical judgment in chronic heart failure management. However, this approach should aim for individualization of the treatment strategy. Likewise, several conceptual, methodological, and practical limitations of natriuretic peptide-guided therapy conflict with the contemporary strategic approach based on symptoms and echo-guided treatment of chronic heart failure. The clinically significant biological variability of natriuretic peptides result in a lower specificity than expected, higher cost, and slow time-course. In addition, the lack of conclusive scientific evidence over a long-term period of intensive scientific investigations and industry investment may indicate a need for new biological markers or novel combinations for multimarker predictive scores and guided therapy. The chapter considers the potency and clinical advantages of a novel strategic approach for CHF treatment based on serial measurements of biomarkers and creating optimal combinations of biological indicators with an aim to improve clinical outcomes, quality of life, and well-being for patients with cardiac failure.

Keywords

Biomarker-guided therapy, chronic heart failure • Biomarkers • Heart failure • Chronic heart failure • Biomarker-guided therapy • BNP-guided therapy • Brain natriuretic peptide (BNP) • Cost-effectiveness • Definition • Diagnosis and prognosis • Limitations • Natriuretic peptide-guided therapy • Novel biomarkers • Treatment strategy • Clinical endpoints • Serial measurements • Surrogate endpoints

Abbreviations

ACC	American College of Cardiology
ACEI	Angiotensin-converting enzyme inhibitors
ACS	Acute coronary syndrome
ADCHF	Acutely decompensated chronic heart failure
ADHF	Acutely decompensated heart failure
AHA	American Heart Association
ANP	Atrial natriuretic peptide
ARB	Angiotensin receptor blocker
BNP	Brain natriuretic peptide
CABG	Coronary artery bypass grafting
CHF	Chronic heart failure
COPD	Chronic obstructive pulmonary disease
ESC	European Society of Cardiology
LVEF	Left ventricular ejection fraction
MI	Myocardial infarction
PCI	Percutaneous coronary intervention

Definitions

Biological marker-guided therapy of heart failure Achieving the optimal goals of heart failure patients based on a dose-adjusted approach of concomitant medications or use of new procedures and interventions under the control of serial measurements of biological markers.

Biomarker A biomarker is defined as an objectively measured indicator of several biological or pathological processes, pharmacologic responses, and therapeutic interventions that may have diagnostic and predictive values to use these markers as potent surrogate endpoint indicators.

Clinically based heart failure treatment In this traditional approach, the initial choice of drugs, optimal combinations and dosage regimen of remedies, and other procedures and interventions for heart failure are based on the analysis of appropriate signs, symptoms, and clinical response after prescribing.

Echo-guided heart failure management This treatment strategy of heart failure is based on serial measurements of echocardiographic parameters reflected in the global pump and diastolic function to correct the dosing of concomitant medications that are suitable for heart failure treatment.

Hemodynamically guided therapy of heart failure This treatment of heart failure is performed under the control of hemodynamics. Usually, this term is synonymous with echo-guided heart failure management.

Relevance Relevance is the ability of a biomarker to clarify clinically relevant of information that is important for healthcare professionals, public and health policy officials, physicians, and all other stakeholders.

Surrogate endpoint biomarker A surrogate endpoint biomarker is defined as an indicator of clear clinical endpoints in target patient populations only.

Validity Validity is defined as the need of a biomarker to exactly reflect the efficacy and/or utility as a surrogate endpoint.

Introduction

Chronic heart failure is the leading cause of cardiovascular morbidity and mortality worldwide (Santulli 2013). CHF occurs in 1–2 % of the adult population in developed countries; this rate rises to more than 10 % in individuals older than 70 years (Mosterd and Hoes 2007). A timely diagnosis and modern treatment can significantly improve both the short-term and long-term prognosis of this disease (Komajda et al. 2015). However, the expected 5-year survival of the patients after

a first admission for symptomatic chronic heart failure remains low and comparable with cancer, despite all of the advances in modern medicine (Stewart et al. 2001). Even patients who receive optimal chronic heart failure therapy may still experience acutely decompensated chronic heart failure, sudden cardiac death, fatal arrhythmias, and urgent admission due to chronic heart failure or other cardiovascular reasons (Schou et al. 2013). The understanding of chronic heart failure has progressed from the concept of a purely hemodynamic disorder to that of a syndrome resulting from dysfunction in several molecular pathways with mutual interconnections (Liu and Eisen 2014). As a result, the focus of research investigations and clinical care has shifted to the measurement and modification of maladaptive molecular processes (Ahmad and O'Connor 2013). In this regard, significant efforts to identify biological markers that reflect several biochemical processes and the risk of clinical outcomes in CHF patients have been used (Scali et al. 2014; Stienen et al. 2014).

Biomarker Definition

Biomarkers are objectively measured and evaluated as indicators of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Biomarkers Definitions Working Group 2001). Biomarkers may unmask different biological processes that contribute to several innate mechanisms of pathogenesis in heart failure and mediate a patient's response to treatment or procedures (Wang et al. 2012; Vasan 2006). Therefore, some biomarkers are considered to be surrogate end-points with high potency for utilization in the management of primary-care subjects and patients at discharge after acute or acutely decompensated heart failure (Bishu et al. 2012; Braun et al. 2009). An ideal biomarker is precise, accurate, and rapidly available to physicians without equivocal and controversial interpretation (Table 1); it should produce new or additional important information that cannot be surmised from clinical evaluation and may help in decision making, in addition to being low cost (Morrow and de Lemos 2007).

Table 1 Expected requirements for ideal biomarkers

Highly sensitive and specific
Easy to detect with low biological variation
Capable of reflecting appropriate molecular interaction, as well as functional, physiological, and biochemical processes at the cellular level
Capable of indicating an acute response after the drug is given or after injury
Quantitatively describes the level of injury by serial measurements
Closely correlates with the severity of disease and prognosis
Predicts progression and target-organ damage
Probability of risk stratification for new events and readmission
Low cost

The Natriuretic Peptides and Heart Failure

Atrial natriuretic peptide (ANP) and brain (or B-type) natriuretic peptide (BNP) are neurohormones secreted predominantly from cardiomyocytes in response to atrial or ventricular wall stretch and intracardiac volume loading (Ancona et al. 2007). The natriuretic peptides have a fundamental role in cardiovascular remodeling, volume homeostasis, and the response to myocardial injury. BNP is considered to be a counterregulatory hormone to angiotensin II, norepinephrine, and endothelin, having vasodilatorary and diuretic effects (Tsutamoto and Horie 2004). The precursor of BNP is pro-BNP, stored in secretory granules in myocytes. Pro-BNP is split by a protease enzyme into BNP and N-terminal pro-BNP (NT-pro-BNP) (Chen and Burnett 2000). BNP can be easily measured in plasma. The main causes of circulating natriuretic peptide elevation are listed in Table 2. The compensatory activity of the cardiac natriuretic peptide system may be attenuated as mortality increases in chronic chronic heart failure patients with high plasma levels of ANP and BNP (Mant et al. 2008). However, BNP and NT-pro-BNP are more useful than ANP for the diagnosis and management of acute decompensated chronic heart failure (Worster et al. 2008). Among patients with chronic heart failure, concentrations of natriuretic peptides are strongly linked to the presence and severity of structural heart disease and are strongly prognostic in this setting (Nishikimi 2012; Valle et al. 2008). Therefore, an average of BNP and NT-proBNP assay results may relate to structure remodeling and biomechanical stress of heart (Ohtani et al. 2012). Because patients with chronic heart failure and preserved left ventricular ejection fraction (LVEF) usually have

Table 2 The main causes of circulating natriuretic peptide elevation

Cardiac reasons	Noncardiac reasons
Heart failure (acute, acutely decompensated, chronic)	Age > 60 years
ACS/MI	Anemia
Stable CAD	COPD
Ventricular hypertrophy	Renal failure
Cardiomyopathies	Obstructive sleep apnea
Myocarditis	Pulmonary hypertension
Valvular heart diseases	Pulmonary thromboembolism
Pericardial disease	Pneumonia
Atrial fibrillation/flatter	Injury
Cardioversion	Malignancy
Cardiac surgical procedure, including PCI, CABG, and pacemaker implantation	Critical illness
Drug-induced cardiac toxicity (adriamycin, 5-ftoruracil)	Toxic-metabolic insult
	Sepsis
	Burns

ACS acute coronary syndrome, *MI* myocardial infarction, *PCI* Percutaneous Coronary Intervention, *CABG* coronary artery bypass graftingm, *COPD* chronic obstructive pulmonary disease, *CAD* coronary artery disease

Table 3 2013 ACC/AHA clinical practice guideline for heart failure: novel issues for biomarkers in heart failure management

Biomarkers	Target	Patient populations	Class of recommendation	Level of evidences
Natriuretic peptides	To establish or refute heart failure	All patients suspected of having HF, especially when the diagnosis is not certain	I	A
	Predict outcome	Outpatients with HF	I	A
	Guided-based therapy	Outpatients with HF	II a	B
	Diagnostic aim	Inpatients suspected with acute HF	II b	C
Cardiac specific troponins	Stratify at risk	Outpatients with HF	I	A
	Predict outcome	Inpatients suspected with acute, acutely decompensated or chronic ischemic HF		
Galectin-3	Stratify at risk	Outpatients with HF	II b	B
	Predict outcome	Inpatients suspected with acute HF	II b	A

HF heart failure

smaller LV cavities and thicker LV walls when compared with subjects with reduced LVEF, the intensity of biomechanical stress is also lower (Patel et al. 2014). It should be considered that patients with preserved LVEF are more likely to be older and female with obesity and hypertension than heart failure patients with reduced LVEF (Lund et al. 2014; Luchner et al. 2013; Mason et al. 2013). As a result, the circulation level of BNP/NT-proBNP may be detected in lower concentrations than in heart failure subjects with reduced LVEF (Tate et al. 2014). Moreover, heart failure patients with preserved LVEF may be more likely to have undetected circulating levels of BNP/NT-proBNP compared with persons without heart failure (Ohtani et al. 2012).

The current guidelines for chronic heart failure management indicate that evidence supports the use of natriuretic peptides for the diagnosis, staging, hospitalization and/or discharge decisions (Table 3), and identification of patients at risk for clinical events and readmission (Yancy et al. 2013; McMurray et al. 2012). Because about 50 % of individuals with left ventricular systolic dysfunction are asymptomatic, BNP levels have been evaluated for this purpose (Costello-Boerrigter et al. 2006; Wang et al. 2004). Currently, the measurement of plasma concentrations of B-type natriuretic peptide (BNP) or N-terminal pro-B-type natriuretic peptide (NT-pro-BNP) is useful to rule out the diagnosis and to predict the prognosis of patients with ischemic and non-ischemic CHF (Table 4), although it remains unclear whether BNP-guided chronic heart failure therapy is beneficial and economically feasible (Vavuranakis et al. 2012). Clinical utilization of cardiac biomarkers in heart failure is reported in Table 5.

Table 4 Advantages and disadvantages of BNP/NT-proBNP implementation in routine clinical practice

Advantages	Disadvantages
More accurate differential diagnosis of acute dyspnea	High biological variability
Reflects the stage and prognosis of HF	No optimal cut-off points for patients older than 60 years
Easy and reproducible detection	Impossible to differentiate diastolic and systolic types of cardiac dysfunction
Ability to be detected by self-measurement	Underdiagnosed in patients with diastolic dysfunction
Low cost	A drop of 15 % and less within 5–7 days of admission due to acute or acutely decompensated heart failure requires additional consideration of the treatment response

HF heart failure, *BNP* brain natriuretic peptide

Table 5 Clinical utilization of cardiac biomarkers in heart failure

Natriuretic peptides[a]	BNP/NT-proBNP, midregional proBNP, midregional proANP
Cardiac injury biomarkers	Troponin T[a], troponin I[a], fatty acid binding protein
Metabolic markers	Adiponectin, grelin, apelin, leptin, insulin-like growth factor-1, cardiotrophin
Neurohormones	Catecholamines, renin, aldosterone, angiotensin, C-terminal pro-vasopressin, mid-regional pro-adrenomedullin, endothelin, urocortin, urotensin
Proinflammatory biomarkers	hs-CRP, galectin-3[a], TNF-alpha, ST2 protein[a], solubilized ST2 protein receptor, interleukins, Fas (APO-1), myeloperoxidase
Bone-related proteins	Ospeoprotegerin, osteopontin, osteonectin
Renal injury biomarkers	Creatinine, NGAL, cystatin C, KIM-1, L-FABP, cysteine-rich protein
Anemia biomarkers	Hemoglobin, RDW, transferrin, ferritin
Other biomarkers	Myotrophin, mRNA, growth differential factor-15, collagen peptides, matrix metalloproteinases, tissue inhibitors of matrix metalloproteinases, circulating endothelial-derived apoptotic microparticles, circulating mononuclear progenitor cells

hs-CRP high sensitive C-reactive protein, *mRNA* micro ribonucleic acid, *TNF* tumor necrosis factor, *APO-1* apoptosis antigen-1, *RDW* red cell distribution width, *NGAL* neutrophil gelatinase-associated lipocalin, *KIM-1* kidney injury molecule-1, *L-FABP* liver-type fatty acid binding protein
[a]Incorporated in clinical practice guidelines

The Principles of Natriuretic Peptide-Guided Therapy in Chronic Heart Failure

Standard chronic heart failure care may substantially improve outcomes in patients affected by the disorder. Unfortunately, the physical signs and symptoms of heart failure lack diagnostic sensitivity and specificity, and medication doses proven to

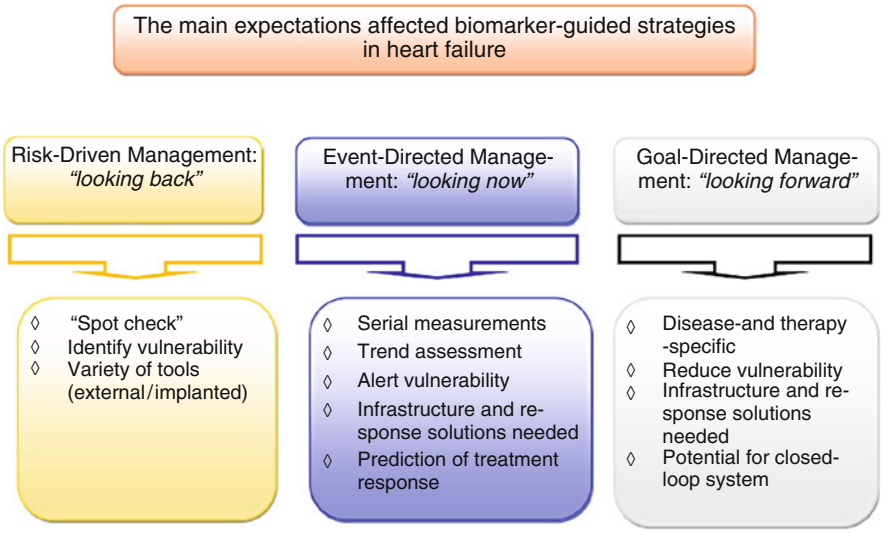

Fig. 1 The main expectations of biomarker-guided strategies in heart failure. This figure shows the principles of biomarker-guided therapy in heart failure that are considered to be suitable for achieving beneficial results (Adapted from data produced by Samara and Tang (2011))

improve mortality in clinical trials are often not achieved (Saremi et al. 2012). Biomarker-guided strategies for heart failure may have some advantages that are usually absent in symptom-based treatment approaches and echo-based strategies (Fig. 1).

Natriuretic peptide-guided chronic heart failure therapy has been given a recommendation in US chronic heart failure guidelines to achieve guideline-directed medical therapy (Class IIa) and possibly improve outcomes (Class IIb). Other clinical practice guidelines (including those from the European Society of Cardiology) are awaiting results from emerging clinical trial data (Yancy et al. 2013; McMurray et al. 2012). Biomarker-guided chronic heart failure trials indicate that the approach improves the quality of care without an excess of adverse events related to more aggressive management (Adams et al. 2010). Additionally, a favorable reduction in the concentration of BNP and NT-pro-BNP may be seen during treatment of chronic heart failure, with parallel improvement in short- and long-term prognosis. Given these issues, there is increasing interest in harnessing cardiovascular biomarkers for clinical applications to more effectively guide diagnosis, risk stratification, and further therapy (Fiuzat et al. 2013). The evidence for their use in monitoring and adjusting drug therapy is less clearly established (Vavuranakis et al. 2012). It may be possible to realize an era of personalized medicine for chronic heart failure care in which therapy is optimized and costs are controlled and, probably, reduced (Ahmad and O'Connor 2013).

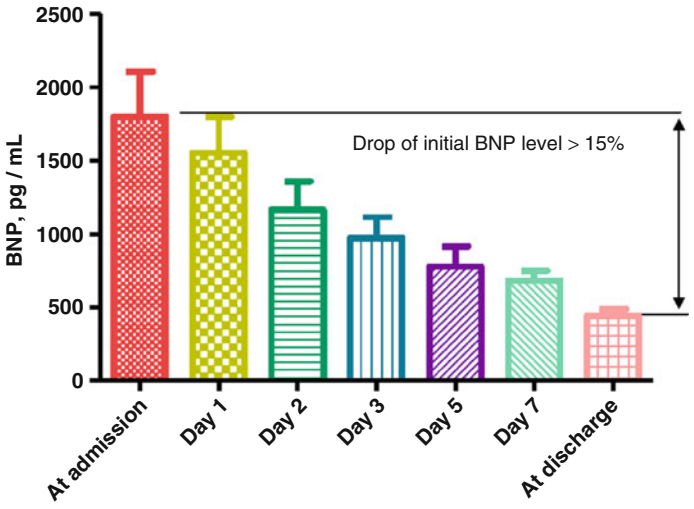

Fig. 2 Schematic trend of decreasing BNP plasma levels in patients with acutely decompensated heart failure. The plot shows a principal trend of decreasing BNP plasma levels with beneficial treatment strategy among inpatients with acutely decompensated heart failure. The data were pooled to obtain a mean and SEM (as *bars*) (The plot was constructed with data adapted from Bayes-Genis et al. (2004) and Kazanegra et al. (2001))

Serial Natriuretic Peptide Measurements as a Useful Predictive Tool in Chronic Heart Failure Management

The natriuretic peptides are important tools to establish diagnosis and prognosis for chronic heart failure patients. With the application of therapies for chronic heart failure, changes in both BNP and NT-pro-BNP parallel the benefits of chronic heart failure therapy that might be applied (Troughton et al. 2013). Among patients admitted with acutely decompensated chronic heart failure (ADCHF), patients who experienced complications were more likely to have much smaller changes (typically a 15 % decrease) in values of NT-pro-BNP compared to those who survived (about a 50 % decrease in NT-pro-BNP values from day 1 to day 7) (Bayes-Genis et al. 2004). Changes in the BNP level during early aggressive treatment have been closely associated with falling pulmonary wedge pressure in patients treated for decompensated CHF (Kazanegra et al. 2001). Overall, it has been asserted that serial measurements of natriuretic peptides could help to modulate more accurately the intensity of drug treatment in patients with chronic heart failure (Januzzi and Troughton 2013). Short-term therapeutic studies of inpatients have largely resulted in a statistically significant decline in BNP and NT-pro-BNP with clinical evidence of patient improvements (Wu 2006). However, serial BNP measurements may be useful in evaluating heart failure because there is a possibility to overcome the biological variability of natriuretic peptides by assessing such measurements (Fig. 2).

In contrast, many therapeutic studies involving long-term outpatient monitoring have produced changes in BNP/NT-pro-BNP that do not exceed the biologic variances (Wu 2013). Nevertheless, strategy of monitoring NT-pro-BNP and BNP to guide therapy cannot be universally advocated because there are still several open questions about the presumed role of natriuretic peptide-guided pharmacologic adjustment as a valuable strategy in this setting (De Vecchis et al. 2013a, b; Miller et al. 2005). Changes in serial BNP levels during the admission of the patients with acutely decompensated heart failure may be predictive of the clinical outcome; however, BNP has not been compared with other parameters, echocardiographic performances (even LVEF), and end points combined in-hospital deaths and post-discharge events (Cheng et al. 2001). In this study, patients had very high levels of BNP and no significant changes of circulating biomarkers during admission were found. Thus, the probability for a decrease of BNP/NT-pro-BNP plasma levels may be associated with the severity of heart failure and, probably, coexisting comorbidities. The so-called obesity paradox suggests that the presence of plasma BNP levels in patients with heart failure may be low when obesity is present (Adamopoulos et al. 2011). When the diagnostic utility of biomarkers for heart failure in older subjects in long-term care were examined, it was found that copeptin (ADM), MR-pro-ADM, and MR-pro-ANP, as well as common signs and symptoms, had little diagnostic value in comparison with BNP (Mason et al. 2013).

A trend of decreasing BNP/NT-pro-BNP plasma level may be a more important factor than the peak level of biomarkers (De Vecchis et al. 2013a, b). It was found that survivors had lower circulating levels of pre-discharged BNP than subjects who died (Ito et al. 2012). In fact, the biological variability of BNP/NT-pro-BNP plasma levels and close relation of circulating levels of biomarker with age, renal function, and comorbidities (such as obesity and diabetes) are the main limitations for the implementation of serial monitoring of BNP/NT-pro-BNP in routine clinical practice.

Continued BNP Home Monitoring in Heart Failure Patients

The hypothesis that adding a BNP level assay to a home monitoring regimen might add significant value in the early detection of heart failure decompensation in stable subjects after discharge was tested in the Heart Failure Assessment with BNP in the Home (HABIT) trial (Maisel et al. 2013). Using a finger-stick test (Alere HeartCheck System) that was specifically designed for the home monitoring of BNP levels in heart failure patients, an upward trend was found to correspond with an increased a risk of early readmission due to ADHF after discharge. Conversely, a downward BNP level trend indicated a risk decrease. Thus, the home monitoring of BNP in stable heart failure patients after discharge may provide sufficient information about the risk of early readmission within 30 days. The assessment of more durable continued monitoring efficacy is desirable to understand whether a novel option is beneficial.

Results of the Most Important Clinical Trials on BNP-Guided Therapy

The use of plasma levels of natriuretic peptides to guide the treatment of patients with chronic heart failure has been investigated in a number of randomized controlled and retrospective clinical trials; however, the results were controversial and the benefits have been high variable. It was found that BNP-guided therapy was not better than an expert's clinical assessment for beta-blocker titration in chronic heart failure patients (Beck-da-Silva et al. 2005). A retrospective study was dedicated to the assessment of serial BNP levels in patients receiving hemodynamically guided therapy for severe chronic heart failure (O'Neill et al. 2005). In patients with severe heart failure, BNP levels did not accurately predict serial hemodynamic changes, including left ventricular ejection fraction (LVEF) and left ventricle dimensions. In the Pro-BNP Outpatient Tailored Chronic Heart Failure Therapy (PROTECT) study, patients treated with biomarker-guided care also had improved quality of life and significantly better reverse remodeling on echocardiography compared with patients who received standard care (Januzzi 2012). A multicenter randomized pilot trial (STARBRITE) tested whether outpatient diuretic management guided by BNP and clinical assessment resulted in longer survival and no hospitalization over 90 days compared with clinical assessment alone (Shah et al. 2011). There was no significant difference in the number of days alive and not hospitalized, change in serum creatinine, or change in systolic blood pressure. A BNP strategy was associated with a trend toward lower blood urea nitrogen; BNP strategy patients received significantly more angiotensin-converting enzyme inhibitors (ACEI), beta-blockers, and the combination of ACEI or angiotensin receptor blocker (ARB) plus beta-blockers (Shah et al. 2011). Not all investigators have confirmed that morbidity and mortality are improved in chronic heart failure patients receiving treatment guided by BNP levels, although significantly better clinical outcomes in BNP responders in comparison with non-responders were reported (Karlström et al. 2011).

The long-term prognostic impact of a therapeutic strategy using plasma brain natriuretic peptide levels was evaluated in the STARS-BNP Multicenter Study (Jourdain et al. 2007). A total of 220 New York Heart Association functional class II to III patients considered to be optimally treated with ACE inhibitors, beta-blockers, and diuretics by chronic heart failure specialists were randomized to medical treatment according to either current guidelines (clinical group) or a goal of decreasing BNP plasma levels <100 pg/ml (BNP group). The primary combined end point was chronic heart failure-related death or hospital stay for chronic heart failure. During the 15-month follow-up period, significantly fewer patients reached the combined end point in the BNP group (Jourdain et al. 2007). The results were mainly obtained through an increase in ACEI and beta-blocker adjusted dosages. Later, the TIME-CHF trial found that, in contrast to chronic heart failure with reduced LVEF, NT-pro-BNP-guided therapy may not be as beneficial in chronic heart failure patients with preserved LVEF (Maeder et al. 2013).

Although patients do not always improve after the implementation of BNP-guided strategy, the heterogeneous results of natriuretic-peptide guided therapy for chronic heart failure were confirmed by several meta-analyses (Li et al. 2013; Savarese et al. 2013). There was a significantly decreased risk of all-cause mortality and chronic heart failure readmission in the BNP-guided therapy group. Age and baseline BNP are the major determinants of chronic heart failure readmission when analyzed using meta-regression. In the subgroup analysis, chronic heart failure readmission significantly decreased in patients younger than 70 years or with higher baseline BNP ($\geq$2,114 pg/mL). When a separate assessment of variables was performed, it was found that NT-pro-BNP-guided therapy significantly reduced all-cause mortality and chronic heart failure-related hospitalization but not all-cause admission. However, BNP-guided therapy did not significantly reduce all-cause mortality, chronic heart failure-related admission, or all-cause admission. It was concluded that BNP-guided therapy did not significantly reduce mortality or morbidity. On the other hand, improved all-cause mortality and CHF-related admission rates were found in BNP-guided therapy cohorts.

Changes in follow-up circulating BNP levels versus peak BNP levels at admission or discharge may be able to stratify CHF patients at risk. The optimal population of these subjects might be an inpatient cohort with ADCHF at admission. Overall, data indicate a close association between BNP on the fifth day after admission due to ADCHF and cardiovascular risk. A marked decrease of circulating BNP may be a strong predictor of a decreased risk of death or new hospitalization, as well as other chronic heart failure-related clinical events. However, clinical trials have been shown that BNP-guided therapy in outpatients was associated with a similar risk of death and/or CHF-related hospitalization compared to a conventional clinical approach (De Vecchis et al. 2013a; Jourdain et al. 2007). Among outpatients with previous ADHF, a substantial improvement in cardiovascular event rates could not be demonstrated in patients treated with BNP-guided therapy compared with those undergoing the usual symptom-guided treatment. The question addressed to inpatients with ADHF is still unresolved and likely requires more investigation (De Vecchis et al. 2013b). On the other hand, some experts believe that novel biomarkers are needed for ADHF instead of natriuretic peptide, such as procalcitonin, ST2 protein, mid-regional ANP, galectin-3, copeptin, and probably fibroblast growth factor (Fig. 3). However, whether serial measurements of these marker levels improve prediction among patients with ADHF when compared with a traditional approach is still not understood.

Cost-Effectiveness of Natriuretic Peptide-Guided Therapy of CHF

Chronic heart failure management strategies have been shown to reduce re-hospitalizations and mortality, but the costs of treatment may cause concern in the current cost-conscious clinical setting. Overall, contemporary chronic heart failure management programs are under increasing pressure to demonstrate their cost-effectiveness in comparison with other approaches to improving patient

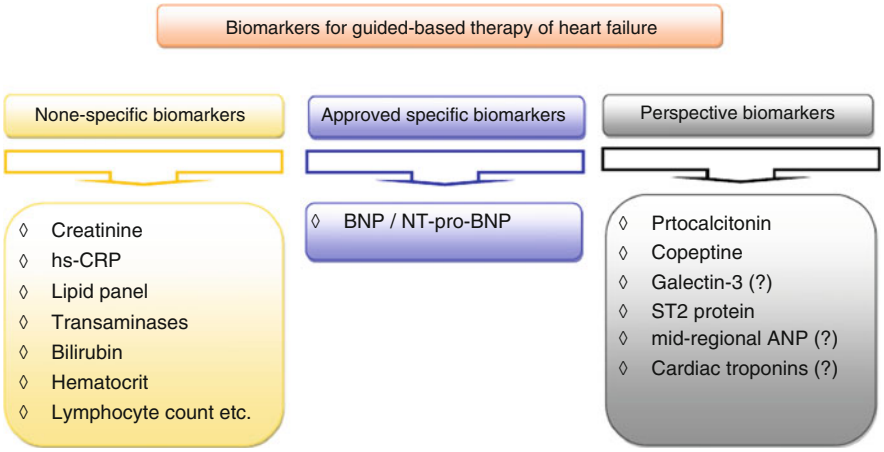

Fig. 3 Biomarkers for guided therapy of heart failure. *Abbreviations: hs-CRP* high sensitive C-reactive protein, *BNP* brain natriuretic peptide, *ANP* atrial natriuretic peptide

outcomes (Turner et al. 2008; Gonseth et al. 2004; Lambrinou et al. 2012). Risk predictive scores (e.g., The Seattle Heart Failure Model) that are based on combination of demographics, symptoms and signs of CHF, and several biomarkers (creatinine, lymphocyte count) significantly predict the survival of subjects with cardiac failure, as well as reduce medical resource use and costs (Levy et al. 2006). Online calculators allow physicians to unmask their knowledge around the risk and prognosis of the subjects observed (O'Connor et al. 2009). It is unclear whether the implementation of biological markers incorporated into risk scores has effective economic utility? It should be investigated if creating a biomarker risk predictive score is a more powerful tool to stratify the chronic heart failure subjects at risk when compared with contemporary predictive models.

Studies have shown that an introduction of BNP measurement in CHF management may be cost effective (Morimoto et al. 2004; Siebert et al. 2006). It was found that the optimal use of NT-pro-BNP guidance could reduce the use of echocardiography by up to 58 %, prevent 13 % of initial hospitalizations, and reduce hospital days by 12 % (Siebert et al. 2006). Moreover, NT-pro-BNP-guided assessment was associated with a 1.6 % relative reduction of serious adverse event risk and a 9.4 % reduction in costs, translating into savings of $474 per patient compared with standard clinical assessment. When a new disease management comparing usual care to home-based nurse care and a home-based nurse care group was investigated, it was concluded that NT-pro-BNP-guided chronic heart failure specialist care in addition to home-based nurse care was cost effective and cheaper than standard care, whereas home-based nurse care was cost neutral (Adlbrecht et al. 2011). Thus, BNP-guided chronic heart failure therapy may be considered as a highly effective strategy to minimize expenditures of the health care system for patients with chronic heart failure.

Limitations of the Natriuretic Peptide-Guided Therapy of CHF

Although the pooling of data derived from clinical trials demonstrates an overall effect of slightly significant improvement in clinical outcomes with a natriuretic peptide-guided approach, there are some relatively large studies that failed to document a significant clinical improvement in terms of mortality and morbidity using a natriuretic peptide-guided strategy (De Beradinis and Januzzi 2012). On the one hand, compared with standard management, biomarker-guided care appears to be cost effective, may improve patient quality of life, and may promote reverse ventricular remodeling. However, randomized clinical trials and real-world practice have affected the implementation of natriuretic peptide-guided therapy. On the other hand, the limitation of standard care strategies is evident from the suboptimal uptake and application of proven therapies documented in chronic heart failure registries (Komajda et al. 2005). Certain subgroups, such as the elderly and subjects at low to moderate cardiovascular risk, may respond in a less vigorous manner to the approach of a natriuretic peptide-guided strategy. In certain studies, patients treated with biomarker-guided care had superior outcomes when compared with standard heart failure management alone, particularly in younger populations, in patients with left ventricular systolic dysfunction, and when substantial reductions in natriuretic peptides were achieved in association with biomarker-guided care (McMurray et al. 2012). This may reflect the effects of age on chronic heart failure therapy. Therefore, subjects at different cardiovascular risk may have different responses to natriuretic peptide-guided therapy. Overall, a novel approach based on biomarker serial measurements requires serious adaptation in real clinical practice (Schou et al. 2013).

Novel Biomarker-Guided Approaches in the Management of CHF

Galectin-3 and ST2 protein, which reflect fibrosis and inflammation status, have been approved by the Food and Drug Administration as predictive biomarkers for heart failure patients (Carrasco-Sánchez and Páez-Rubio 2014). Unlike BNP/NT-pro-BNP, circulating galectin-3 and soluble ST2 protein concentrations are not affected by obesity, age, atrial fibrillation, or the etiology of heart failure (Piper et al. 2014; Lok et al. 2013). Therefore, both biomarkers have also shown significantly less individual variability over a 1-month time period compared with BNP (Piper et al. 2014; Lok et al. 2013). Although most of the studies involved patients with heart failure and systolic dysfunction, galectin-3 seems to have a more accurate role in heart failure patients with preserved LVEF then with reduced LVEF (Carrasco-Sánchez and Páez-Rubio 2014). Results of the ProBNP Outpatient Tailored Chronic Heart Failure Therapy (PROTECT) study have demonstrated that the serial measurement of circulating galectin-3 adds incremental prognostic information to a conventional predictive score and closely ameliorates the prediction value of cardiac remodeling (Motiwala et al. 2013). Unfortunately, no clear effect of contemporary heart failure treatment on galectin-3 levels was found (Motiwala

et al. 2013). In the Val-HeFT study, baseline galectin-3 was not associated with a risk of all-cause mortality, but an increased biomarker level over time in heart failure patients was independently associated with worse outcomes (Anand et al. 2013). As in the PROTECT and Val-HeFT studies, no beneficial effect of serial measurement on outcomes was determined. The results of the Biomarkers in ACute Heart Failure (BACH) study suggested that the measurement of three biomarkers (MR-proANP, BNP, and NT-proBNP) allows for an increase in the predictive value for combination biomarkers, but the role of the approach in guided-based therapy remained unclear (Richards et al. 2013).

Serial measurements of midregion pro-ANP (MR-pro-ANP) and C-terminal provasopressin (copeptin) in ambulatory patients with heart failure were detected as possible approach for improving prognosis and clinical outcomes (Miller et al. 2012). It is well known that MR-pro-ANP and copeptin are precursor peptides of the natriuretic and vasopressin systems, respectively. As expected, a strategy based on the serial monitoring of MR-pro-ANP and copeptin combined with circulating cardiac troponin T (cTnT) might be advantageous in elucidating and managing outpatients with heart failure at high risk (Miller et al. 2012). The obtained results have shown that MR-pro-ANP, and to a lesser extent copeptin, seem to add support for an incremental value of serial measurements of BNP and cTnT over time (median = 18.9Insert Space instead off ±Insert Space instead off 7.8 months). Finally, this and other data indicate that two different phenotypes of heart failure may be detected using biomarkers: with and without beneficial response after intervention. It is reasonable to believe that biomarker-guided therapy might useful for initial and maintenance therapy of heart failure, as well as the inadequacy of an intervention requiring a dose-adjusted regimen or additional new drugs. However, numerous types of optimal components of biomarker panels for pre-treatment risk stratification and heart failure evolution remain a big question.

These findings have stimulated new attempts to investigate novel biomarkers, often with negative or equivocal results. Cardiac specific troponins were investigated in studies of patients with acute ischemic heart failure and ADHF. Both forms of troponins (cTnT and cTnI) have significantly predicted in-hospital mortality in patients after myocardial infarction, but serial measurements of their concentration did not confirm the ability of standard heart failure treatment to improve survival by reducing troponin levels (Xue et al. 2011; Peacock et al. 2008; Fonarow et al. 2008). However, a rapidly rising level of cTnI during admission was associated with worse outcomes when compared with limited or no increased levels. Overall, targeting a troponin level is possible but rarely achieved. Other novel biomarkers, such as fibroblast-growth factors and procalcitonin, may be indicators of reparation processes, but their use in guided therapy of heart failure is currently only in the proof-of-concept stage. Although procalcitonin seems to be an attractive option, evidence is only available for acute dyspnea, acute heart failure, and ADHF (Travaglino et al. 2014; Naffaa et al. 2014).

Novel biomarkers have shown great promise and stimulated much interest in their validation for acutely decompensated heart failure. However, there are no data about their superiority to conventional biomarkers, such as natriuretic peptides, in

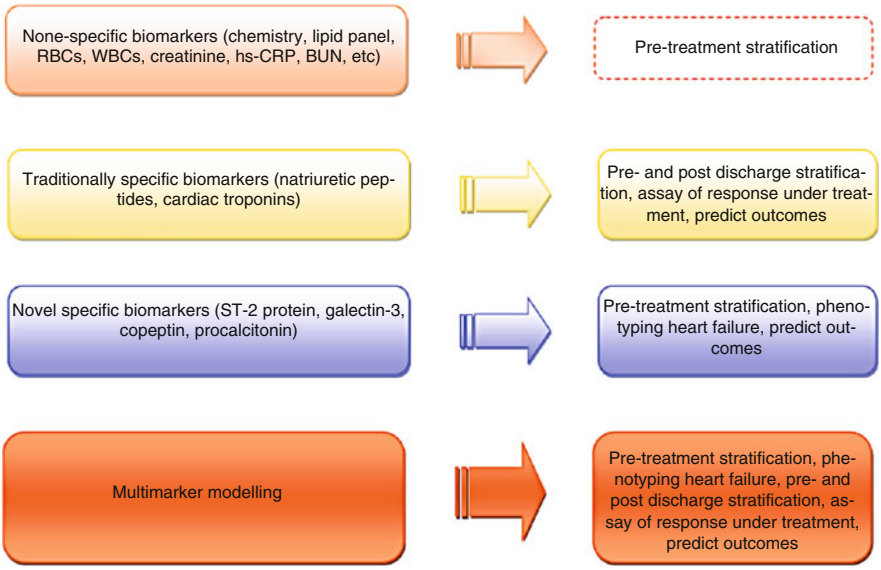

Fig. 4 Future possibilities for the implementation of biomarker-based strategies in heart failure treatment. *Abbreviations*: *RBCs* red blood cells, *WBCs* white blood cells, *hs-CRP* high sensitive C-reactive protein

postdischarge patients with chronic heart failure. In fact, biomarkers that indicate the phenotype of heart failure (ST-2 protein, galectin-3) are not suitable for serial monitoring in guided therapy. Conversely, natriuretic peptides are more optimal for serial monitoring. It has been postulated that future biomarker modelling will use a multimarker approach to stratify patients at risk and reassay therapy response (Fig. 4).

Potential Applications for Prognosis of Other Diseases or Conditions

BNP and NT-pro-BNP have good diagnostic and prognostic performance for heart failure. The serial measurement of circulating BNP/NT-pro-BNP may provide important and sufficient information about heart failure evolution under treatment. As expected, individualized treatment of heart failure based on biomarker monitoring may be more effective and safe then contemporary strategies. High biological variation of BNP/NT-pro-BNP concentration, as well as relation to renal function, aging, and comorbidities, should be considered as the main limiting factors for the implementation of serial measurements in routine clinical practice. Because there is a significant difference in the results of studies dedicated to biomarker-guided therapy of heart failure, serial measurements need to be interpreted carefully. Novel biomarkers (ST-2 protein, galectin-3, copeptin, procalcitonin) have shown great

promise and stimulated much interest in their validation for acutely decompensated heart failure; however, their superiority to conventional biomarkers, such as natriuretic peptides, in postdischarge patients with chronic heart failure is still not understood. A biomarker-based strategy may lead to an era of personalized medicine for chronic heart failure care in which therapy is optimized and costs are reduced.

Conclusion

Studies have suggested that a strategy of standard-of-care management together with a goal to suppress BNP or NT-pro-BNP concentrations leads to greater application of guideline-derived medical therapy and is well tolerated. In addition, a variety of novel (ST-2 protein, galectin-3, copeptin, procalcitonin) or already used (natriuretic peptides) biomarkers have been tested in small trials for heart failure management. Larger randomized clinical trials should be conducted in the future, with high statistical power to address the unresolved issues of natriuretic peptide-guided therapy in chronic heart failure. The future of heart failure management will probably involve an algorithm to use clinical assessment along with a biomarker-guided approach.

Summary Points

- This chapter focuses on serum-based biomarkers that are essential for guided management of patients with chronic heart failure.
- Biomarker-guided therapy of heart failure is an attractive aspect of this approach aimed at individualizing of the treatment strategy.
- Biomarker use for heart failure patients can help to determine the initial diagnosis, stratify patients at risk of acute or acutely decompensated heart failure, and monitor patients during the chronic phase to prevent readmission.
- Natriuretic peptide can guide therapy to prevent the onset of heart failure in at-risk primary care patients and likely assess hospital discharge readiness for patients with acutely decompensated heart failure.
- The concentrations of novel biomarkers, such as galectin-3 and soluble ST2 protein, are not affected by obesity, age, atrial fibrillation, or the etiology of heart failure. BNP/NT-pro-BNP may affect the probability of equivocal interpretation and controversial opinions.
- Phenotyping of heart failure biomarkers (ST-2 protein, galectin-3) is probably not suitable for serial monitoring in guided therapy.
- Circulating neurohumoral biomarkers (natriuretic peptides, copeptin, and procalcitonin) are more suitable and useful for biomarker-guided strategies in heart failure (Tables 1, 2, 3, 4, and 5).

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Part II

Circulating and Body Fluid Biomarkers

Biomarkers of the Extracellular Matrix and of Collagen Fragments

5

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_5

Abstract

A great body of evidence has shown that extracellular matrix (ECM) alterations are present in the major types of cardiac diseases: ischemic heart disease, heart disease associated with pressure overload, heart disease associated with volume overload, and intrinsic myocardial disease or cardiomyopathy. Collagen, types I and III, is the principal structural protein found in the myocardium, and its pro- or telopeptides are released into the circulation during the course of cardiovascular diseases. Therefore, these peptides may reflect collagen synthesis and breakdown and also represent a much more useful tool to address ECM changes from a distance. Clinical trials have been performed during recent years to examine the usage of these peptides as diagnostic or prognostic biomarkers in heart failure (HF) patients. This review aims to summarize published data concerning cardiac ECM and its circulating biomarkers. Studies that focused on collagen metabolism-related biomarkers in patients with HF are analyzed. Finally, limitations associated with the clinical use of the aforementioned biomarkers are also discussed.

Keywords

Biomarker • Collagen • Galectin • Extracellular matrix • Heart failure • Extracellular matrix • Peptides

Abbreviations

BNP	Brain natriuretic peptides
CMR	Cardiac magnetic resonance
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
HF	Heart failure
LGE	Late gadolinium enhancement
MMPs	Matrix metalloproteinases
PICP	Carboxy terminal propeptide of procollagen type I
PIIICP	Procollagen type III carboxy-terminal propeptide
PIIINP	Procollagen type III amino-terminal propeptide
PINP	Amino-terminal propeptide of procollagen type I
TIMP	Tissue inhibitors of metalloproteinases

Key Facts of Extracellular Matrix-Derived Biomarkers in Heart Failure

- Changes in the quantity and quality of the extracellular matrix, including the collagen network, is a main pathophysiologic feature of left ventricular remodeling in heart failure.
- Pro- and telopeptides of collagen types I and III are released into the circulation during extracellular matrix metabolism.
- Several clinical studies in humans have identified a possible value of these biomarkers regarding diagnosis, prognosis, or treatment management in heart failure.
- There are numerous limitations in the laboratory assessment of these biomarkers mainly their biological variation, their non-specificity for heart failure pathology, and their dependence of various demographic, clinical, or treatment confounders.
- Up to date their application in everyday clinical practice is limited.

Definitions

Analytic *Analytic* related to the process of laboratory measurements.

Dilated cardiomyopathy Dilated cardiomyopathy is a primary disease of the myocardium in which the whole myocardium is thinned and unable to pump efficiently without any cause.

Extracellular matrix *Extracellular matrix* is a complex mixture of collagen fibrils, elastin, cells including fibroblasts and macrophages, macromolecules such as glycoproteins, and glycosaminoglycans together with other molecules such as growth factors, cytokines, and extracellular proteases.

Fibrosis *Fibrosis* is the formation of excess fibrous connective tissue in an organ or tissue in a reparative or reactive process.

Heart failure *Heart failure* is a syndrome in which the heart is unable to pump sufficiently to maintain blood flow to meet the needs of the body.

Hypertrophic cardiomyopathy *Hypertrophic cardiomyopathy* is a primary disease of the myocardium in which a portion of the myocardium is hypertrophied without any obvious cause.

Propeptide *Propeptide* is a protein precursor, an inactive protein that can be turned into an active form by posttranslational modification (usually by cleavage).

Telopeptide Telopeptide is an amino acid sequence (normally at one or more ends) that have a function in building or conforming a protein and are proteolytically removed at maturity.

Variation *Variation* is the extent to which or the range in which a thing varies.

Ventricular remodeling *Ventricular remodeling* refers to the changes in size, shape, structure, and physiology of the heart after injury to the myocardium.

Introduction

The present chapter is an update of a previous relevant review from our group (Chalikias and Tziakas 2014) adding recently published collagen biomarkers data (up to November 2014), incorporating new data regarding the clinical use of galectin and commenting further on analytical issues as far as the practical use of these markers is concerned (adapted by permission; Elsevier License number 3543691010039).

It has been over 25 years since Karl Weber focused our attention on cardiac extracellular matrix (ECM) regarding myocardial remodeling in various cardiovascular diseases based on pioneering work by his group and others (Weber 1989). A great body of evidence has shown that ECM alterations are present in four major types of cardiac diseases: ischemic heart disease, heart disease associated with pressure overload, heart disease associated with volume overload, and intrinsic myocardial disease or cardiomyopathy (Lopez et al. 2010).

ECM consists of a macromolecular network of fibers. Collagen is the principal structural protein, whereas a basement membrane, proteoglycans, glycosaminoglycans and bioactive signaling molecules are also significant constituents (Bowers et al. 2010). The ECM network is a metabolically active structure in the sense that there is a continuous turnover of its elements, mainly a dynamic balance between synthesis and degradation of collagen, which is estimated to be from 80 to 120 days (Laurent 1987).

In the past, the myocardial collagen fraction was determined in cardiac biopsies. However, during the past 10 years, there has been a growing interest in noninvasive methods to detect cardiac collagen. Late gadolinium enhancement (LGE) or extracellular volume fraction are two cardiac magnetic resonance (CMR) imaging techniques to detect fibrotic areas in the heart (Schelbert et al. 2014). Given the dynamic nature of ECM, the costs and time consumption of CMR, the ECM-related changes cannot be assessed by classical imaging alone (Shirani and Dilsizian 2008). Therefore, circulating biomarkers that reflect collagen synthesis or degradation might be much more useful tool to address ECM changes from a distance, especially if these changes should be followed on multiple occasions over time by the bedside (Weber 1997).

This chapter aims to summarize published data concerning cardiac ECM and its circulating biomarkers. Studies that focused on collagen metabolism-related biomarkers in patients with heart failure (HF) are discussed.

ECM Metabolism

The cardiac ECM is predominantly composed of fibrillar collagen type I (85 %) and type III (11 %) (De Jong et al. 2011; Medugorac and Jacob 1983). Type I has a poor specificity, forms thicker fibers, and has a high degree of cross-linking between the fiber, thus conferring tensile strength and resistance to stretch and deformation. Type III is more specific to the heart, has a relatively small diameter, and provides resilience and elasticity (Bishop and Laurent 1995; Zannad et al. 2010; Bower et al. 2006). Furthermore, small amounts of types IV and V are observed in the basement membrane of the myocytes, perivascular, and in the pericellular space (De Jong et al. 2011; Eghbali and Weber 1990). As mentioned above proteoglycans, glycosaminoglycans and bioactive signaling molecules represent less abundant elements of the ECM.

Collagen turnover is regulated by fibroblasts and by fibroblasts differentiated to myofibroblasts (Lopez et al. 2010; Wynn 2008). These cells respond to mechanical stretch, wall stress, autocrine and paracrine factors generated locally (such as angiotensin II) and growth factors (such as transforming growth factor-β or connective tissue growth factor), and hormones derived from the circulation (e.g., aldosterone) (Lopez et al. 2010). In addition, a number of pro-inflammatory cytokines (e.g., tumor necrosis factor-α, interleukin-1, and interleukin-6) secreted by monocytes and macrophages also influence the function of fibroblasts and myofibroblasts (Lopez et al. 2010). The responses of these cells to all the aforementioned factors include changes in their rates of proliferation and migration and modifications in their capacity to synthesize and secrete fibrillar collagen precursors (namely, the two more abundant subtypes present in the heart: procollagen types I and III), as well as enzymes that process procollagen precursors to mature collagen able to form fibrils and fibers (e.g., procollagen proteinases and lysyl oxidase), enzymes that degrade collagen molecules within fibers (e.g., matrix metalloproteinases [MMPs]), and signaling molecules that regulate the interaction of the extracellular matrix with parenchymal cells (e.g., matricellular proteins) (Lopez et al. 2010).

Collagen Synthesis

The fibrillar collagen is synthesized as a pre-procollagen, a pro-a-collagen chain within fibroblasts or myofibroblasts. In the endoplasmic reticulum, three pro-a-chains form a triple helix structure, known as procollagen (De Jong et al. 2011; Lopez et al. 2001). All fibrillar procollagen types initially contain two propeptides: the amino (N)-propeptide and the carboxy (C)-propeptide. Once the procollagen is

localized to the ECM, these propeptides are cleaved by proteinases in a 1:1:1 stoichiometry (De Jong et al. 2011; Risteli and Risteli 1995). Cleavage of the propeptides is a prerequisite for the formation of type I and III collagen fibers. This holds true for the carboxy-terminal propeptide of procollagen type I (PICP) and possibly for the amino-terminal propeptide of procollagen type I (PINP) (De Jong et al. 2011; Risteli and Risteli 1990; Jensen and Host 1997). On the other hand, the carboxy-terminal and amino-terminal propeptides of collagen type III (PIIICP and PIIINP, respectively) are not completely cleaved during the conversion of procollagen type III into collagen type III, remaining to some extent in the final fiber. Moreover, fibril formation will still occur, together with the incorporation of these propeptides and thus also being released during fiber degradation (De Jong et al. 2011; Risteli and Risteli 1990; Jensen and Host 1997). After cleavage of the propeptides, the triple helix chain will form large collagen fibrils together with other collagen chains cross-linked by pyridinium and deoxy-pyridinium containing bonds (Fig. 1) (Jensen and Host 1997).

The removal of procollagen type I carboxy-terminal propeptide (PICP) and procollagen type III carboxy-terminal propeptide (PIIICP) is accomplished by procollagen C-proteinases. The N-terminal propeptides (PINP and PIIINP) are cleaved by members of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin type I repeats) family (De Jong et al. 2011; Trackman 2005). After cleavage of the propeptides, the propeptides are released into the blood, either directly or via the lymphatics (De Jong et al. 2011; Risteli and Risteli 1995). Finally, they are degraded by the liver. Elimination of PICP occurs via endocytosis mediated by the mannose receptor, whereas PINP and PIIINP are removed via scavenger receptors (De Jong et al. 2011; Risteli and Risteli 1995, 1990). However, elimination of the propeptides might also occur via the kidneys and via urine (De Jong et al. 2011; Risteli and Risteli 1995). The N-terminal propeptides of collagen type I or III(PINP and PIIINP) and the C-terminal propeptides (PICP and PIIICP) are used as markers of collagen type I or III synthesis.

Collagen Degradation

The degradation of collagen fibers is mediated by the matrix metalloproteinase (MMP) family of enzymes that can be inhibited by direct interaction with naturally occurring, specific tissue inhibitors of metalloproteinases (TIMP-1 to TIMP-4) (Lopez et al. 2010; Malemud 2006). Interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8), and collagenase-3 (MMP-13) initiate the digestion of collagens by hydrolyzing the peptide bond following a glycine residue located at a distance of three quarters of the collagen molecule length from the amino-terminal extreme. The resulting one-quarter carboxy-terminal telopeptide released by the action of MMP-1 on collagen type I (ICTP, 12 Kda) is found in an immunochemically intact form in blood (Lopez et al. 2010; Risteli et al. 1993). A stoichiometric ratio of 1:1 exists between the number of collagen type I molecules degraded and of ICTP molecules released, and the amount of ICTP that reaches the circulation is

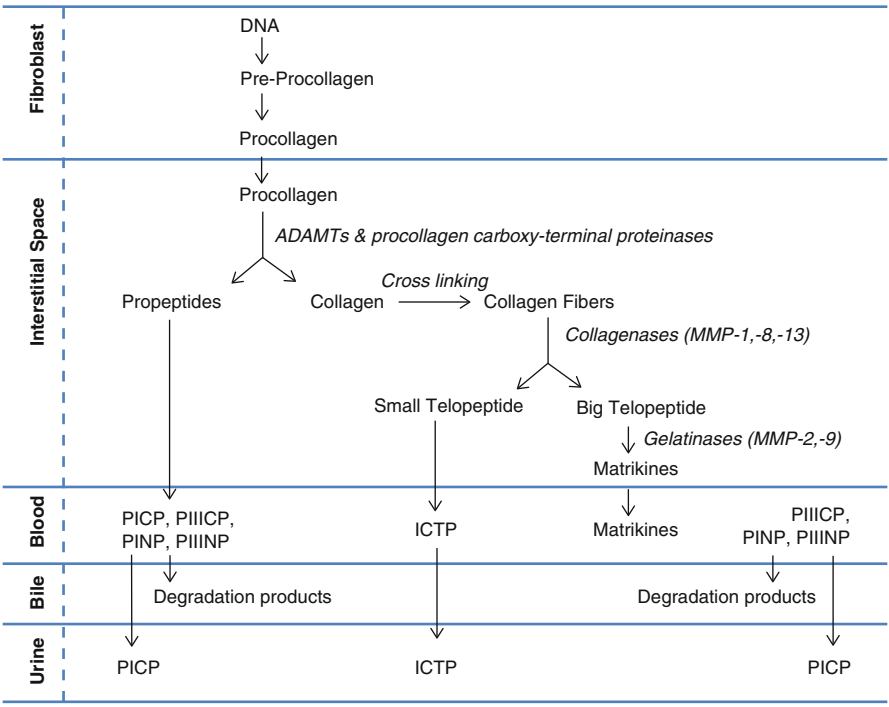

Fig. 1 Synthesis and degradation of collagens I and III. *PICP* procollagen type I carboxy-terminal propeptide, *PINP* procollagen type I amino-terminal propeptide, *ICTP* collagen type I cross-linked carboxy-terminal telopeptide, *PIIICP* procollagen type III carboxy-terminal propeptide, *PIIINP* procollagen type III amino-terminal propeptide, *MMP* matrix metalloproteinase (Adapted from Chalikias and Tziakas (2014) by permission; Elsevier License number 3538261127514; Zannad et al. 2010; Lijnen et al. 2012)

proportional to the amount of fibrillar collagen degraded (Lopez et al. 2010; Sternlicht and Werb 2001). Therefore, ICTP may qualify as an index of MMP-1 – dependent collagen type I degradation. We have to underscore the possibility that during collagen degradation, peptides (PINP, PIIINP, PIIICP) produced during synthesis may be released into the circulation as these peptides may have been incompletely cleaved from procollagen. The resulting three-quarter fragment amino-terminal telopeptide released by MMP-1 from the collagen molecule is further degraded by MMP-2 and MMP-9 or gelatinases (Lopez et al. 2010). The final fragmented matrix peptides or matrikines released by the action of these enzymes have biological activities in the regulation of collagen metabolism and angiogenesis (Lopez et al. 2010; Maquart et al. 1988). However, the stoichiometry of these matrikines relative to the degradation of the larger collagen type I telopeptide is unknown (Lopez et al. 2010) (Fig. 1). Besides these collagenases and gelatinases, MMP-3 (stromelysin) and MMP-7 are expressed in the human myocardium. Stromelysin is able to cleave basement membranes, proteoglycans, and elastin.

MMP-7 has many substrates, for example, collagen fibrils, proteoglycans, and fibronectin, because this MMP lacks specific substrate recognition sites (Spinale 2007).

Galectin

Galectins are carbohydrate-binding proteins involved in the regulation of satellite cell signaling, immunity, and cancer. Galectin-3 (aka MAC-2 Ag) is an approximately 30 kDa glycoprotein expressed in the nucleus and mitochondria that has a carbohydrate-recognition-binding domain of approximately 130 amino acids that enables the binding of β-galactosides (Cooper 2002; Dumic et al. 2006; Krześlak and Lipińska 2004). Galectin-3 as a paracrine protein directs cell adhesion, activation, chemoattraction, growth and differentiation, upregulation of the cell cycle, and apoptosis (Henderson and Sethi 2009). In the myocardium, galectin-3 assists transforming growth factor β (TGF-β) to increase cell cycle (cyclin D1) of fibroblasts and of myofibroblasts which results in their proliferation and synthesis of procollagen type I (McCullough 2014).

There is ample experimental evidence suggesting a link between galectin expression and collagen synthesis (fibrosis). The upregulation of myocardial galectin-3 and its association with fibrosis has been demonstrated in a rat model of HF-prone hypertensive hearts (Sharma et al. 2004), interferon 6-induced murine chronic active myocarditis and cardiomyopathy (Reifenberg et al. 2007), rat streptozotocin-induced diabetic cardiomyopathy (Thandavarayan et al. 2008), and rat angiotensin II-induced hypertension (Sharma et al. 2008); in several studies, this upregulation was associated with the concomitant activation of macrophages. Finally, several studies showed that the co-infusion of Ac-SDKP – an antifibrotic agent – along with galectin-3 not only inhibited fibrosis and inflammation but also alleviated cardiac dysfunction (Sharma et al. 2008; Liu et al. 2009).

In summary, production of galectin-3 from local pericytes, mast cells, and macrophages within the myocardium induces resident fibroblasts and myofibroblasts to produce procollagen which is irreversibly cross-linked to collagen-generating cardiac fibrosis (McCullough 2014).

ECM-Related Biomarkers and Cardiovascular Diseases

Because biomarkers of collagen metabolism present in blood are not cardiac specific, the problem of how to demonstrate their relationship with the lesions of the collagen network present in cardiac diseases emerges. To address this issue, it has been proposed that a given circulating biomarker must be investigated to answer a number of questions before it is considered as biomarker of ECM (Table 1) (De Jong et al. 2011; Gonzalez et al. 2009).

Table 1 Questions to be answered before a circulating molecule can be considered as a biomarker of the myocardial collagen network

Is there an association between its expression or mechanism of production in the myocardium and its blood concentration?
Is there a positive gradient from its concentration in coronary sinus blood toward its concentration in peripheral vein blood, thus proving its main cardiac origin?
Are there associations of its concentration in blood with the myocardial histopathological alteration and the disturbances of LV morphology and function under study?
Do its levels vary in parallel with the changes in the above myocardial alteration and LV disturbances induced by treatment?
Does it exhibit adequate diagnostic performance (e.g., sensitivity and specificity) to detect the histopathological alterations under study?
Is its measurement reproducible, cheap, and easily accessible to clinicians?
Does it add additional information next to existing tests?
Does it improve management of patients?

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C-Terminal Propeptide of Collagen Type I (PICP)

PICP, a 100 kDa propeptide, is a marker of the synthesis of collagen type I, and in contrast to PINP and PIIINP, PICP is cleaved off the procollagen without exception in a 1:1 stoichiometric fashion (Risteli and Risteli 1990). Furthermore, it has been observed that in the setting of steady-state production by extracardiac sources, PICP detected in peripheral blood from patients with hypertensive heart disease is mainly of cardiac origin because a positive gradient exists for its serum concentration from the coronary sinus toward antecubital vein in these patients but not in normotensive subjects (Lopez et al. 2010; Querejeta et al. 2004). Serum PICP as well as coronary PICP is positively correlated with the myocardial collagen content in hypertensive heart disease (Querejeta et al. 2004; Lopez et al. 2004) as well as in idiopathic dilated cardiomyopathy (Izawa et al. 2005).

Table 2 summarizes findings of clinical studies assessing PICP levels in HF patients of various etiologies (Lijnen et al. 2012). In patients with HF, a higher serum PICP level was found in comparison with control subjects, except in the study of Alla et al. (2006), of Plaksej et al. (2009), of Schartzkopff et al. (2002) and of Lombardi et al. (2003).

N-Terminal Propeptide of Collagen Type I (PINP)

PINP is the 70-kDa N-terminal peptide of collagen type I and can be used as a marker for collagen type I synthesis (De Jong et al. 2011; Risteli and Risteli 2002). Table 3 summarizes observations regarding PINP levels in clinical studies. However, no significant difference is shown in serum PINP levels between controls and hypertrophic cardiomyopathy patients (Lombardi et al. 2003), HF patients

Table 2 PICP in patients with heart failure of various etiologies

Reference	Studied condition	Main findings
Gonzalez et al. (2010)	Hypertension + HF (EF > 50 %)	↑ in both patient groups
Qurejeta et al. (2000)	Hypertension + HF (EF > 45 %)	↑
Querejeta et al. (2004)	Hypertension (EF < 50 %)	↑
Diez et al. (2002)	Hypertension (EF > 50 %)	↑
Plaksej et al. (2009)	Hypertension + HF	=
Lopez et al. (2004)	HF (EF > 45 %)	Myocardial collagen ∼PICP
Martos et al. (2007)	HF (diastolic) (EF > 50 %)	↑
Barasch et al. (2009)	HF (diastolic and systolic) (EF > 55 % and EF < 55 %)	=, associated with diastolic HF,
		= between groups
Alla et al. (2006)	HF (EF < 35 %)	=
Lombardi et al. (2003)	HCM	=
Schartzkopff et al. (2002)	DCM (EF < 45 %)	=
Izawa et al. (2005)	DCM	Myocardial collagen ∼PICP
Ho et al. (2010)	HCM	↑
Müller-Brunotte et al. (2007)	Hypertension + HF (EF > 50 %)	↑
Jiménez-Navarro et al. (2005)	HF (systolic vs diastolic) (EF < 45 % and EF > 45 %)	↑ in both patient groups
Löfsjögård J et al. (2014)	HF (systolic, EF ≤ 45 %)	Independently related to increased BNP levels and relative wall thickness

Adapted from Chalikias and Tziakas (2014) by permission; Elsevier License number 3538261127514; De Jong et al. 2011; Lijnen et al. 2012), *BNP* brain natriuretic peptide, *DCM* dilated cardiomyopathy, *EF* ejection fraction, *HCM* hypertrophic cardiomyopathy, *HF* heart failure, *PICP* C-terminal propeptide of collagen type 1, ↑ increase, = no significant difference

(Alla et al. 2006), and hypertensive patients with or without diastolic HF (Martos et al. 2007). In contrast, in hypertrophic cardiomyopathy patients, a negative correlation between PINP and echocardiographic markers of passive diastolic function was observed suggesting that increased PINP levels could be used as surrogates for deteriorating passive diastolic function (Lombardi et al. 2003). Furthermore, a recent study showed that in hypertensive patients with asymptomatic heart failure as shown by brain natriuretic peptide (BNP) levels, PINP levels were linearly associated with increasing BNP levels (Phelan et al. 2012). Risteli and Risteli (1995) in a relevant review cited a number of flaws about using PINP levels as a marker for collagen type I synthesis

Table 3 PINP in patients with heart failure of various etiologies

Reference	Studied condition	Main findings
Martos et al. (2007)	HF (diastolic) (EF > 50 %)	=
Alla et al. (2006)	HF (EF < 35 %)	=
Lombardi et al. (2003)	HCM	=; inversely associated with diastolic function
Kaye et al. (2013)	HF (EF < 25 %)	=
Phelan et al. (2012)	Hypertension + asymptomatic HF	Associated with BNP
Lin et al. (2009)	HF (EF < 45 %)	No association with EF or NYHA class
Chatzikyriakou et al. (2009)	HF (EF < 45 %)	↓ with resolution of symptoms
Ellims et al. (2014)	HCM	=

Adapted from Chalikias and Tziakas (2014) by permission; Elsevier License number 3538261127514; De Jong et al. 2011; Lijnen et al. 2012), *BNP* brain natriuretic peptide, *EF* ejection fraction, *HCM* hypertrophic cardiomyopathy, *HF* heart failure, *NYHA* New York Heart Association, *PINP* N-terminal propeptide of collagen type 1, ↓ decrease, = no significant difference

(De Jong et al. 2011). First, there is a delay between the release of PINP, compared with the release of PICP (Risteli and Risteli 1995; De Jong et al. 2011). Second, like PIIINP, PINP may not always be removed from the collagen, which suggests that PINP levels might give an unreliable value (Risteli and Risteli 1995; De Jong et al. 2011). Third, they suggested that uncleaved PINP could degrade into a monomer. This monomer might still react in some PINP assays, resulting in an overestimation of collagen type I synthesis (Risteli and Risteli 1995; De Jong et al. 2011).

N-Terminal Propeptide of Collagen Type III (PIIINP)

PIIINP is the 42-kDa N-terminal propeptide of procollagen type III (De Jong et al. 2011; Risteli and Risteli 2002) and is widely used as a marker for collagen type III synthesis. Patients with dilated cardiomyopathy (Klappacher et al. 1995), hypertrophic cardiomyopathy (Lombardi et al. 2003), and HF (Alla et al. 2006; Barasch et al. 2009) have higher serum PIIINP levels than do healthy controls. This elevated level of serum PIIINP was also shown in hypertensive patients with diastolic HF, compared with in hypertensive patients without diastolic HF (Martos et al. 2007). Table 4 summarizes observations regarding PIIINP levels in clinical studies. Whether PIIINP levels indeed reflect the synthesis of cardiac collagen type III remains unclear. The N-terminal domain of collagen type III is sometimes removed incompletely, resulting in the incorporation of PIIINP in the collagen fibers (De Jong et al. 2011; Risteli and Risteli 1990). This may lead to an underestimate of the synthesis of collagen type III. However, Klappacher et al. (1995) in a seminal study showed that circulating PIIINP levels correlated adequately ($r = 0.784$) to the myocardial level of collagen type III, using human cardiac biopsies. Furthermore, this peptide is also shown to be related to several echocardiographic parameters

Table 4 PIIINP in patients with heart failure of various etiologies

Reference	Studied condition	Main findings
Diez et al. (1995)	Hypertension (EF > 50 %)	↑
Klappacher et al. (1995)	DCM (EF < 40 %)	↑
Biolo et al. (2009)	HF (EF < 40 %)	↑ if RAP > 15 mmHg
Martos et al. (2007)	HF (diastolic) (EF > 50 %)	↑
Barasch et al. (2009)	HF (diastolic and systolic) (EF > 55 % and EF < 55 %)	↑, associated with diastolic HF, = between groups
Alla et al. (2006)	HF (EF < 35 %)	↑
Lombardi et al. (2003)	HCM	↑
Plaksej et al. (2009)	Hypertension + HF	↑ in NYHA III and IV
Zile et al. (2011)	HF (diastolic) (EF > 55 %)	↑
Shah et al. (2013)	Cardiogenic shock	↑
Phelan et al. (2012)	Hypertension + asymptomatic HF	Associated with BNP
Kaye et al. (2013)	HF (EF < 25 %)	↑; associated with capillary wedge pressure
Bishu et al. (2012)	HF (diastolic vs systolic) (EF > 50 % vs EF < 50 %)	=
de Denus et al. (2012)	HF (diastolic vs systolic) (EF > 55 % vs EF < 30 %)	Associated with reduced EF
Collier et al. (2011)	Hypertension (EF > 50 %)	Associated with diastolic dysfunction
Lin et al. (2009)	HF (EF < 45 %)	Association with NYHA class
Biolo et al. (2010)	HF (EF < 45 %)	=
Poulsen et al. (2000)	MI	↑
Sato et al. (1997)	DCM	↑
Ellims et al. (2014)	HCM	=

Adapted from Chalikias and Tziakas (2014) by permission; Elsevier License number 3538261127514; De Jong et al. 2011; Lijnen et al. 2012), *DCM* dilated cardiomyopathy, *EF* ejection fraction, *HCM* hypertrophic cardiomyopathy, *HF* heart failure, *MI* myocardial infarction, *NYHA* New York Heart Association, *PIIINP* N-terminal propeptide of collagen type III, *RAP* right atrial pressure,↑ increase, = no significant difference

(De Jong et al. 2011). PIIINP levels are inversely related to the systolic and diastolic functions in patients with hypertensive heart disease (Plaksej et al. 2009; Diez et al. 1995; Rossi et al. 2004), left ventricular end-diastolic diameter in patients with hypertrophic cardiomyopathy (Lombardi et al. 2003), and positively associated

Table 5 ICTP in patients with heart failure of various etiologies

Reference	Studied condition	Main findings
Klappacher et al. (1995)	DCM (EF < 40 %)	↑
Barasch et al. (2009)	HF (diastolic and systolic) (EF > 55 % and EF < 55 %)	↑, associated with diastolic HF, = between groups
Lombardi et al. (2003)	HCM	↑
Plaksej et al. (2009)	Hypertension + HF	↑ in NYHA IV
Zile et al. (2011)	HF (diastolic) (EF > 55 %)	↑
Schartzkopff et al. (2002)	DCM (EF < 45 %)	↑
Martos et al. (2007)	HF (diastolic) (EF > 50 %)	↑
Jiménez-Navarro et al. (2005)	HF (systolic vs diastolic) (EF < 45 % and EF > 45 %)	= in both patient groups
Phelan et al. (2012)	Hypertension + asymptomatic HF	Associated with BNP
Collier et al. (2011)	Hypertension (EF > 50 %)	Associated with diastolic dysfunction
Chatzikyriakou et al. (2009)	HF (EF < 45 %)	↑ with resolution of symptoms
Löfsjögård J et al. (2014)	HF (systolic, EF ≤ 45 %)	independently related to increased BNP levels
Ellims et al. (2014)	HCM	=

Adapted from Chalikias and Tziakas (2014) by permission; Elsevier License number 3538261127514; De Jong et al. 2011; Lijnen et al. 2012), *BNP* brain natriuretic peptide, *DCM* dilated cardiomyopathy, *EF* ejection fraction, *HCM* hypertrophic cardiomyopathy, *HF* heart failure, *NYHA* New York Heart Association, *ICTP* collagen type I cross-linked carboxy-terminal telopeptide,↑ increase, = no significant difference

with right atrial pressure in patients with HF (Biolo et al. 2009). In contrast, Wang et al. (2007) found no association between echocardiographic parameters of left ventricular structure and function with PIIINP levels in ambulatory individuals.

Collagen Type I Carboxy-Terminal Telopeptide (ICTP)

The small C-terminal telopeptide of collagen type I (ICTP, 12 kDa) is cleaved by collagenase in a 1:1 stoichiometric way, during the breakdown process of collagen type I fibrils (Risteli and Risteli 2002). ICTP is a marker of the degradation of collagen type I. Increased serum ICTP levels are observed in HF patients of various etiologies (Table 5). Klappacher et al. (1995) observed increased serum ICTP levels in patients with dilated cardiomyopathy, compared with healthy controls. In patients with dilated cardiomyopathy, the increased ICTP levels might reflect enhanced collagen breakdown due to dilation of the ventricles (De Jong et al. 2011). However, in the same study, ICTP levels also correlated significantly with the myocardial levels of collagen type I ($r = 0.603$). This is a contradictory observation because

ICTP is thought to reflect collagen type I degradation. Furthermore, in hypertrophic cardiomyopathy patients (Lombardi et al. 2003), in hypertensive cardiomyopathy patients (Plaksej et al. 2009), and in HF patients with diastolic dysfunction (Martos et al. 2007; Barasch et al. 2009), ICTP levels are also elevated suggesting a shift of the collagen equilibrium toward collagen type I breakdown. This is also a remarkable observation because these cardiomyopathies generally show an increase in myocardial stiffness as a consequence of collagen deposition (Risteli and Risteli 2002).

Various explanations have been proposed in order to elucidate these contradictory observations. It has been shown that myocardial fibrosis is to be accompanied by collagen degradation and matrix metalloproteinase activation, suggesting that collagen degradation is a prerequisite for collagen synthesis, accumulation, and fibrosis (Jugdutt 2003). Additionally, myocardial structural changes seen with HF are characterized with a shift of the collagen phenotype and in specific from type I to type III indicating that the release of ICTP into circulation reflects a first step in ventricular fibrosis (collagen type I degradation) before collagen accumulation (collagen type III synthesis) (Jugdutt 2003; Yamamoto et al. 2002). Besides the shift in collagen type, increased cross-linking and thickening of the existing collagen fibers might also be an explanation for the contradictory results of elevated ICTP in patients with diastolic dysfunction (Risteli and Risteli 2002; Jugdutt 2003). Whereas the pathophysiological meaning of isolated ICTP measurement still remains to be established, this peptide can be used in combination with PICP to assess collagen type I turnover on the whole (Lopez et al. 2010). In support of the above hypothesis, animal studies have proposed that the circulating PICP:ICTP ratio may be an index of the degree of coupling between the synthesis and the degradation of collagen type I (Diez et al. 1996). Of interest this hypothesis has been reproduced in studies in humans showing that the ratio is associated with the severity of myocardial fibrosis in patients with hypertensive cardiomyopathy (Diez et al. 2002; Martos et al. 2007). Moreover, Schwartzkopff et al. (2002), observed that serum ICTP concentrations were elevated in patients with dilated cardiomyopathy, whereas PICP levels were not changed significantly, supporting the hypothesis that in patients with dilated cardiomyopathy collagen breakdown is solely increased. In contrast, these results were not observed (ICTP levels were elevated, whereas the PINP and PICP levels were not altered significantly) in similar studies in hypertrophic cardiomyopathy patients (Lombardi et al. 2003), in hypertensive cardiomyopathy patients (Plaksej et al. 2009), and in HF patients with diastolic dysfunction (Barasch et al. 2009). However, PIIINP levels were increased in all previously discussed patient groups, (Plaksej et al. 2009; Lombardi et al. 2003; Martos et al. 2007; Barasch et al. 2009) which implies a shift in the collagen type I/III ratio (Risteli and Risteli 2002).

C-Terminal Propeptide of Collagen Type III (PICP)

Currently to the present review, there are no available data on serum PIIICP in heart failure of any etiology.

Galectin-3

Galectin-3 has been found to be significantly upregulated in the hypertrophied hearts of patients with aortic stenosis and in the plasma of patients with acute and chronic HF.

Sharma et al. (2004) provided the first report in human subjects. They studied ventricular biopsies from patients with aortic stenosis with preserved or depressed ejection fraction and showed that galectin-3 was upregulated in the biopsies from patients with depressed ejection fraction. Van Kimmenade et al. (2006) published the first clinical study that evaluated the potential diagnostic role of galectin-3 as a plasma biomarker in heart failure. In the acute heart failure setting, galectin-3 levels were characterized by a diagnostic accuracy of 0.72 (area under the curve) (van Kimmenade et al. 2006). The optimal cutoff of galectin-3 in this study was 6.88 ng/mL, which resulted in a reasonable sensitivity of 80 % but a poor specificity of 52 % (van Kimmenade et al. 2006). In the same clinical setting of acute heart failure, Shah et al. (2010) showed that galectin-3 concentrations were higher in patients with acutely decompensated heart failure and also associated with echocardiographic markers of ventricular function.

Several clinical studies have provided evidence that galectin-3 levels are also increased in the chronic heart failure setting. In the COACH trial, patients in New York Heart Association (NYHA) classes III and IV had higher galectin-3 levels, whereas BNP and NT-proBNP levels were also higher when galectin-3 levels were higher (de Boer et al. 2011). However, there was no relationship between galectin-3 and echocardiographic or hemodynamic indexes, and in specific no differences were found between preserved or reduced ejection fraction (de Boer et al. 2011). In data from DEAL-HF, a randomized study that enrolled 232 patients with chronic HF, 114 patients (49 %) had galectin-3 plasma concentrations above the upper limit of the normal cutoff value of 17.7 ng/mL (Lok et al. 2010). Similarly, there was no correlation with LV ejection fraction (Lok et al. 2010). In the HF-ACTION study, in ambulatory patients with chronic HF and systolic dysfunction, higher galectin-3 concentrations were associated with NYHA class, higher NT-proBNP levels, longer exercise duration on cardiopulmonary exercise test, shorter distances in 6-min walk distance, and lower maximal oxygen consumption (Felker et al. 2012). Tang et al. (2011) who evaluated chronic HF and advanced decompensated HF in 178 patients found that higher galectin-3 concentration was associated with poor functional capacity (NYHA class), marginally higher NT pro-BNP levels, poor renal function, and more advanced HF. However, no associations were observed with echocardiographic or hemodynamic indices (Tang et al. 2011). A recent study by Lin et al. (2009) described the relation between serum galectin-3 and markers of extracellular matrix turnover in chronic HF patients. Galectin-3 was correlated with PIIINP, TIMP-1, and MMP-2 but not with PINP and LV ejection fraction (Lin et al. 2009). Finally, a small study by Milting et al. (2008) involving 55 patients with end-stage HF with the need for mechanical circulatory support showed that galectin-3 levels were increased compared with controls.

Taken together, from available clinical data, plasma and/or serum galectin-3 is increased in acute and chronic heart failure and is associated with poor functional capacity and circulating collagen metabolism biomarkers; however, its usefulness in detecting heart failure or adding incremental value (over currently used clinical correlates and NT-proBNP) in the diagnostic work-up of heart failure remains unclear (de Boer et al. 2010).

Potential Application to Prognosis

Using collagen metabolism-related biomarkers as a diagnostic tool seems very promising. In many studies, a difference in biomarker levels is shown between patients and controls. To some extent, the biomarker levels can also predict cardiac events or assess prognosis, may be used to evaluate or guide therapies, and also may reflect the effect of standard therapies on cardiac remodeling (Risteli and Risteli 2002). However, there is still much variety between different studies.

Prognosis

The association of circulating ECM metabolism-related biomarkers and prognosis has been assessed in several studies (Table 6). In summary, in the heart failure clinical setting, there is ample evidence for the prognostic ability of PIIINP and ICTP, whereas there is scarcity or paucity of data regarding PINP or PICP and PIIICP, respectively.

Although the relation between PIIINP levels and myocardial collagen type III has to be elucidated more thoroughly, circulating PIIINP is thought to predict cardiac events and mortality. Sato et al. (1997) were the first to investigate the relation between PIIINP levels and cardiac event prognosis. They observed a positive correlation between high PIIINP serum concentrations (>0.8 U/mL) and cardiac events after a follow-up of 500 days in patients with DCM (Sato et al. 1997). Cicoira et al. (2004) confirmed the increased mortality risk of high PIIINP levels in patients with mild to moderate HF (EF < 45 %). Patients with plasma PIIINP levels >4.7 μg/L had a worse outcome regarding survival (Cicoira et al. 2004). Also Klappacher et al. (1995) observed an increased mortality risk in HF patients with PIIINP levels >7 mg/L. In a sub-study of the randomized aldactone evaluation study, in patients with severe HF, Zannad et al. (2000) have shown that serum PIIINP levels >3.85 μg/L have a significant negative correlation with survival and hospitalization-free survival. In patients with acute myocardial infarction, Poulsen et al. (2000) has shown that serum PIIINP levels >5 μg/L are an independent predictor of cardiac death and development of congestive HF. A recent study (Cardiovascular Health Study) by Agarwal et al. (2014) has shown that in 2,568 older adults that were followed up for 14 years, PIIINP was associated with total cardiovascular risk (hazard ratio per SD = 1.07; 95 % CI 1.01–1.14) and heart failure risk (HR per SD = 1.08; CI, 1.01–1.16) but not with myocardial infarction or stroke. Another

Table 6 Circulating collagen metabolism biomarkers and prognosis

Reference	Study n	Studied condition	Marker	End point	Follow-up	Independence	Main findings
Sato et al. (1997)	21	DCM	PIINP	Mortality	500 days	No	↑ risk with >0.8 U/L
				Hospitalization			
Cicoira et al. (2004)	101	HF (EF < 45 %)	PIIINP	Mortality	6 months	Yes	↑ risk with >4.7 μg/L
				Hospitalization			
Klappacher et al. (1995)	41	DCM	PIIINP	Mortality	230 days	Yes	↑ risk with >7 μg/L
			ICTP				↑ risk with >7.6 μg/L
Zannad et al. (2000)	261	HF (EF < 35 %)	PIIINP	Mortality	1 year	No	↑ risk with >3.85 μg/L
				Hospitalization			(placebo)
Kitahara et al. (2007)	156	HF (EF < 50 % and >50 %)	ICTP	Mortality	2 years	Only in EF > 50 %	↑ risk with >7.3 ng/mL
				Hospitalization			
Poulsen et al. (2000)	47	MI	PIIINP	Mortality	1 year	Yes	↑ risk with >5 μg/L
				Readmission			
Manhenke et al. (2011)	233	MI	PIIINP	Mortality	2 years	Yes	↑ risk with ICTP >4.4 μg/L
			PINP	HF			
			ICTP				
Eschalier et al. (2013)	246	MI	PIIINP/ ICTP	Mortality	3 years	Yes	↑ risk with ratio ≤1
				Hospitalization			
Agrinier et al. (2013)	125	HTN	PIIINP	Mortality	5.5 years	Yes	=
				Hospitalization			

(*continued*)

Table 6 (continued)

Reference	Study n	Studied condition	Marker	End point	Follow-up	Independence	Main findings
Lopez-Andres et al. (2012)	260	HF (EF < 30 %)	PIIINP	Mortality	1.5 years	Yes	↑ risk with PIIINP
			PINP	Hospitalization			
Krum et al. (2011)	334	HF (EF > 50 %)	PINP	Mortality	6 months	Yes	=
			PIIINP	Hospitalization			
			ICTP				
Ho et al. (2009)	131	HF (EF < 40 %)	PIIINP	Mortality	240 days	Yes	↑ risk with >6.3 μg/L
				Hospitalization			
Radauceanu et al. (2008)	1,009	HF (HF < 30 %	PIIINP	Mortality	1 year	Yes	↑ risk
				Hospitalization			
Tziakas et al. (2012)	196	HF	ICTP	Mortality	1 year	Yes	↑ risk with > 0.35 ng/ml
		(EF < 55 %)					
Barthelemy et al. (2009)	432	MI	ICTP	Mortality	1 year	Yes	↑ risk with > 5.4 μg/L
				CV events			
Agarwal et al. (2014)	2,568	Older adults	PIIINP	CV events	14 years	Yes	↑ risk CV disease, HF
Bascher et al. (*2011*)	880	Older adults	PIIINP	CV events	≈12 years	Yes	↑ risk MI, HF, and CV death
			CITP				
Chang et al. (2014)	105	HF (EF ≤ 50 %)	PIIINP	Mortality	≈3 years	No	↑ risk for death

Adapted from Chalikias and Tziakas (2014) by permission; Elsevier License number 3538261127514; De Jong et al. 2011; Lijnen et al. 2012), *CV* cardiovascular, *DCM* dilated cardiomyopathy, *EF* ejection fraction, *HTN* hypertensive cardiomyopathy, *HF* heart failure, *ICTP* collagen type I cross-linked carboxy-terminal telopeptide, *MI* myocardial infarction, *PINP* N-terminal propeptide of collagen type 1, *PIIINP* N-terminal propeptide of collagen type III, ↑ increase, = no significant difference

analysis of the same database by Barasch et al. (2011) showed that in 880 participants of the same cohort, in unadjusted and adjusted models, PIIINP levels were associated with myocardial infarction, incident HF, hospitalization for HF, and cardiovascular and all-cause mortality. In contrast, PICP levels were not associated with outcomes in the same cohort (Barasch et al. 2011). Furthermore, Chung et al. (2014) showed in a relatively small cohort study that PIIINP were predictive of mortality in HF patients, however only in unadjusted models.

Regarding the prognostic ability of PINP levels, there is only one study in published literature showing that increased PINP levels are associated with poor prognosis in HF patients. Krum et al. (2011) showed that for each 10 μg/L increase in PINP levels, the hazard ratio for the primary end point was 1.09 (95 % CI, 1.052–1.13; $P < 0.0001$). However, this association was ameliorated in multivariable analysis. Furthermore, the incidence of ventricular tachycardia episodes in implantable cardiac defibrillator (ICD) recipients seems to be associated with increased PINP levels (Blangy et al. 2007). Unfortunately, no other studies have investigated the predictive value of PINP levels.

Several studies have assessed the prognostic value of ICTP levels in HF patients. Klappacher et al. (1995) observed an increased risk of mortality when serum ICTP levels were higher than the cutoff value of 7.6 mg/L. In patients with HF, ICTP levels may predict cardiac events independently, especially in HF patients with preserved left ventricular systolic function (Kitahara et al. 2007). An analysis of the Cardiovascular Health Study by Barasch et al. (2011) showed that ICTP levels were associated with myocardial infarction, incident HF, hospitalization for HF, cardiovascular, and all-cause mortality. Similar to PINP, ICTP levels can predict shock therapy before implantation in patients with ICD (Kanoupakis et al. 2010). In patients with an acute myocardial infarction, Manhenke et al. (2011) demonstrated that ICTP, but not PINP or PIIINP, was an independent predictor for total and cardiovascular mortality.

In some studies, the ratio of collagen synthesis levels to collagen degradation markers had significant prognostic value. Eschalier et al. (2013) showed that in myocardial infarction, patients with PIIINP/ICTP ≤ 1, measured 1 month after myocardial infarction, had the highest risk of developing a primary composite event (cardiovascular death or hospitalization for worsening heart failure) during a 3-year follow-up.

Finally, collagen metabolism markers have been associated with soft end points regarding prognosis in HF. In specific, Chatzikyriakou et al. (2012) showed that ICTP levels are associated with quality of life in HF patients.

On the other hand of the spectrum, galectin-3 measured in blood has been shown to predict the development of all-cause mortality and heart failure in the general population, identify increased risk for de novo heart failure and progressive loss of renal filtration function in healthy middle-aged adults, predict cardiac failure in patients after acute coronary syndromes, and aid in the prognosis of both systolic and diastolic heart failure for the outcomes of hospitalization and death (McCullough et al. 2014) (Table 7).

Table 7 Prognostic ability of galectin-3 in clinical studies

Reference	Study n	Studied condition	End point	Follow-up	Independence	Main findings
de Boer et al. (2012)	7,968	General population	All-cause	10 years	Yes	↑ risk
			CV mortality			All-cause mortality
Ho et al. (2012)	3,353	General population	Incident HF all-cause mortality	8.1 years	No/yes	↑ risk all-cause mortality
						↑ risk incident HF
Grandin et al. (2012)	200	ACS	Incident HF	2 years	Yes/no (for added BNP)	↑ risk incident HF
van Kimmenade et al. (2006)	599	Acute or decompensated HF	Death Hospitalization	60 days	Yes	↑ risk death
						↑ risk hospitalization
Shah et al. (2010)	115	Acute dyspnea	Mortality	4 years	Yes	↑ risk mortality
de Boer et al. (2011)	592	Chronic HF	Death	18 months	Yes	↑ risk death + hospitalization
			Hospitalization			
Lok et al. (2010)	240	Chronic HF (NYHA III/IV)	All-cause mortality	12 months	Yes	↑ risk mortality
			Hospitalization			
Felker et al. (2012)	895	Ambulatory chronic HF (EF < 35 %)	All-cause mortality	2.5 years	Yes/no (for added BNP)	↑ risk death + hospitalization
			Hospitalization			

Tang et al. (2011)	178	Chronic HF (EF < 35 %)	All-cause mortality	5 years	Yes	↑ risk mortality
			Hospitalization			Associated with poor renal function
Chang et al. (2014)	105	Chronic HF (EF < 50 %)	Mortality	≈3 years	No	Borderline ↑ risk mortality
Meijers et al. (2014)	902	Chronic HF	Hospitalization	120 days	Yes	↑ risk hospitalization
Djoussé et al. (2014)	924	General population	HF incidence	–	Yes	↑ risk HF incidence
Carrasco-Sánchez et al. (2013)	419	HF with EF >45 %	All-cause mortality	1 year	Yes	↑ risk death + hospitalization
			Hospitalization			
Gullestad et al. (2012b)	1,462	Systolic ischemic HF	CV death, nonfatal MI, or stroke	33 months	No	↑ risk combined end point
Lopez-Andrès et al. (2012)	260	HF with EF < 35 %	All-cause mortality	1.5 years		↑ risk death + hospitalization
			Hospitalization			

ACS acute coronary syndrome, *BNP* brain natriuretic peptide, *CV* cardiovascular, *EF* ejection fraction, *HF* heart failure, *MI* myocardial infarction, *NYHA* New York Heart Association, ↑ increase

Regarding the prognostic ability of galectin in combination with NT-proBNP levels, few studies showed an independent association; however, more studies showed an attenuated or no independent association.

In summary, according to data from clinical studies, measurement of galectin-3 concentration can be integrated into the management of patients with chronic HF (Hrynchyshyna et al. 2013). For individuals with a galectin-3 concentration $\leq$17.8 ng/mL, continuation of usual care is suggested, with periodic outpatient follow-up visits; for those in the 17.9–25.9 ng/mL range which constitutes moderate risk, more intensified care management is suggested, with possibly more frequent visits, medication monitoring, and adjustment. Finally, for those with concentrations $>$25.9 ng/mL or a doubling of galectin-3, there is a very high risk of hospitalization and death over 18 months; accordingly, they should receive particular attention, with optimal care and advanced management strategies (Hrynchyshyna et al. 2013).

Effect of Standard Therapies

Resolution of symptoms in the acute decompensation HF setting or follow-up post an acute myocardial infarction is associated with significant changes in collagen metabolism marker levels. Chatzikyriakou et al. (2009) showed a significant change in ICTP (increase) and in PINP (decrease) levels with resolution of symptoms in acutely decompensated heart failure patients. Furthermore, an animal study corroborated the aforementioned finding by observing changes in left ventricular collagen content and ECM proteins following reversal of hemodynamic overload (Hutchinson et al. 2011). In contrast, Bishu et al. (2012) observed no significant change in PIIINP in acutely decompensated heart failure patients with preserved or reduced ejection fraction in the DOSE trial. Manhenke et al. (2011) demonstrated significant longitudinal changes in PINP and ICTP levels following myocardial infarction with standard therapy. However, no changes were seen for PIIINP levels (Manhenke et al. 2011). In contrast, Poulsen et al. (2000) showed significant changes in PIIINP levels during a 1-year follow-up in myocardial infarction patients. Similar results for ECM-related markers (PINP, PIIINP, ICTP) have been shown in a multicenter prospective study in patients with a first anterior myocardial infarction (Eschalier et al. 2013). Finally, a prospective study in ST elevation myocardial infarction patients has shown a net type I collagen breakdown in the first week following ST elevation myocardial infarction compensated by an early increase in collagen type III synthesis (Manhenke et al. 2014). Following these changes, there is an increase in both type I and III collagen synthesis markers at 2 months and 1 year, indicating a persistent increase in collagen turnover even in these apparently successfully treated patients (Manhenke et al. 2014). Taken into consideration all the above observations, it is logical to hypothesize that if standard treatment in heart failure patients is associated with significant and measurable changes in collagen metabolism markers, therefore these markers could be used to guide or monitor therapy. Indeed seminal studies from our group (Chatzikyriakou et al. 2010; Kampourides et al. 2012) and others

(Zannad and Radauceanu 2005) have shown that circulating levels of ECM-related biomarkers could be used to identify group of HF patients that could benefit more from standard therapies. However, it must be noted that these changes in circulating levels are time dependent regarding the acute event and also disease specific.

Changes in circulating concentrations of ECM-related biomarkers have been demonstrated with a variety of standard treatment in heart failure and hypertensive heart disease. This holds true in some studies for angiotensin-converting enzyme inhibitors (Tziakas et al. 2012; Chatzikyriakou et al. 2010), for angiotensin receptor blockers (Diez et al. 2002; Lopez et al. 2005; Ciulla et al. 2004; Muller-Brunotte et al. 2007), for diuretics (Lopez et al. 2004), for b-blockers (Fukui et al. 2016), and for aldosterone blockers (Zannad et al. 2010; Deswal et al. 2011; Udelson et al. 2010; Iraqi et al. 2009; Li et al. 2009; Mak et al. 2009). However, this effect of treatment on collagen-related biomarkers was not consistently associated with improvement in symptoms, quality of life, or parameters of left ventricular remodeling (Udelson et al. 2010; Mak et al. 2009). In support to this contradiction, there are several studies in published literature suggesting a nonsignificant effect of standard therapies for heart failure on circulating levels of collagen metabolism markers. In specific, studies assessing the effect of treatment with diuretics (Bishu et al. 2012; Anguita et al. 2011), with aldosterone antagonists (Gupta et al. 2014), or with angiotensin receptor blockers (Krum et al. 2011) on collagen-related markers in heart failure patients compared to placebo have demonstrated not significant changes. Under the same notion, response to cardiac resynchronization therapy cannot be shown using circulating biomarkers of collagen metabolism (Lopez-Andres et al. 2012; Umar et al. 2008; Marin et al. 2011; Dong et al. 2011). Finally, on the other edge of the spectrum use of more advanced therapies in heart failure such as left ventricular assist devices (Wu et al. 2012) or surgical ventricular restoration (ten Brinke et al. 2011) has been associated with significant changes in these biomarkers. Despite evidence of the involvement of the sympathetic system in left ventricular hypertrophy, cardiac remodeling, and fibrosis, there is little information on the effects of beta-blocker therapy on the prevention of cardiac fibrosis in experimental models or clinical studies assessing circulating markers of ECM metabolism (Zannad et al. 2010).

Regarding galectin-3, there is conflicting evidence as far as the effect of standard treatment on its circulating concentrations. The PROTECT study showed that standard HF therapies have no clear effects on galectin-3 levels (Motiwala et al. 2013). The CARE-HF data showed that cardiac synchronization therapy had similarly no effect on galectin-3 levels (Lopez-Andres et al. 2012). Furthermore, a recent analysis of the HF-ACTION study showed no evidence of interaction between galectin-3 and treatment effect of mineral corticosteroid antagonists (Fiuzat et al. 2014). Under the same notion, a sub-analysis of Val-HeFT showed that galectin levels could not identify patients that could benefit more from valsartan treatment (Anand et al. 2013).

On the other hand, increased galectin-3 levels identified a subpopulation of CHF patients that could benefit more from spironolactone therapy (Maisel et al. 2014), rosuvastatin use (Gullestad et al. 2012a), and cardiac resynchronization therapy implementation (Stolen et al. 2014; Truong et al. 2014) in several studies.

The COACH study revealed that patients on ACE inhibitors had lower galectin-3 levels (de Boer et al. 2011). In HF patients with preserved ejection fraction considering HF therapy, plasma galectin-3 concentrations at admission were lower in patients receiving beta-blockers and spironolactone comparing with untreated patients (Hrynchyshyna et al. 2013).

Clinical Use Issues of ECM-Related Biomarkers

Laboratory Measurement Issues

All the aforementioned collagen metabolism-related markers can be measured in serum or plasma samples easily and reproducibly with commercially available ELISAs or radioimmunoassays. The specimen type (serum or plasma) may be of importance. For some of these peptides, ethylenediaminetetraacetic acid (EDTA)-anticoagulated plasma was shown to be stable for a longer period of time in room temperature compared to serum (Chubb 2012). Under this notion, some authors recommend that EDTA plasma should be used with rapid centrifugation and either immediate analysis or freezing of the plasma (Chubb 2012). Most recent studies have used serum samples, but the data suggest that if the samples had been processed promptly, this is unlikely to have caused confounding (Chubb 2012). Therefore, when using biomarkers, consequent and adequate procedures of collecting and storage of samples are important, should be taken into account, and should be reported (De Jong et al. 2011; Risteli and Risteli 2002). Having in mind also that different commercial kits for the same biomarker may give variable quantitative results, depending on the antibodies and standards used, it is important to standardize these determinations before applying them to routine clinical practice (Lopez et al. 2010).

An issue of concern is that whereas most of the peptides generated during the processing of procollagen types I and III are found as one antigen form in serum, PINP appears in two different forms: one form corresponding to the whole propeptide and a smaller form that is the product of its degradation (Lopez et al. 2010; Risteli and Risteli 1997). It is thus important to use assays that can identify the intact molecule as the biomarker of interest (Lopez et al. 2010). Another cautionary note when using N-terminal propeptides is that this propeptide can be incorporated in the collagen network, instead of cleavage and elimination via the circulation. When measuring PINP or PIIINP levels, this might give an underestimation of the collagen synthesis (De Jong et al. 2011; Risteli and Risteli 1995). In addition, PIIINP may be modified in circulation. PIIINP can form larger aggregates with other peptides and other PIIINP peptides. It depends on the commercially available kit as to which form will be detected (De Jong et al. 2011; Risteli and Risteli 2002). Regarding galectin-3 measurements, its concentrations were equivalent when measured in serum or EDTA plasma in a recent analytical study (Christenson et al. 2010).

Disease-Specific Issues

The type of disorder seems to play a role in the levels of the biomarkers (Lopez et al. 2010; De Jong et al. 2011; Zannad et al. 2010). Furthermore, a temporal profile in the kinetics of these peptides seems to exist in various diseases and especially in myocardial infarction patients. Hypertensive heart disease, heart failure with preserved systolic function, and hypertrophic cardiomyopathy is thought to be associated with an increase in collagen fibers. These patients are therefore thought to have an increase in biomarkers reflecting the synthesis of collagen, for example, PIIINP, PINP, and PICP (De Jong et al. 2011). However, some studies have shown an increase in collagen markers associated with collagen breakdown (De Jong et al. 2011). This contradiction may be explained by a hypothesis suggesting that collagen breakdown is a prerequisite or a first step before synthesis to occur. Another explanation is that it is only the cross-linking of collagen fibers that is increased rather the collagen synthesis of new fibers per se (Lopez et al. 2012).

On the other hand, dilated cardiomyopathy, heart failure with reduced ejection fraction, and left ventricular remodeling associated with myocardial infarction are hypothesized to be associated with a decrease in collagen content (De Jong et al. 2011). Conversely, several studies did not show a clear change in the equilibrium of collagen turnover in favor of collagen breakdown. In some studies, the equilibrium is even in favor of synthesis. Possible explanations for this contradiction would be that only the cross-links between the fibers are reduced in dilated myocardium or that the equilibrium between collagen types I and III is shifted toward collagen type III in dilated heart failure, a less stiff collagen type than collagen I (De Jong et al. 2011; Janicki and Brower 2002). Another explanation for the increase in the collagen amount in dilated hearts might be increased perivascular collagen, as also shown in patients with systolic heart failure (Lopez et al. 2006; Chatzikyriakou et al. 2008).

Finally, an increased concentration of galectin-3 was found in patients with chronic HF, regardless of etiology and HF typology (with preserved or reduced ejection fraction), and in acute HF, hypertrophic hearts, and aortic stenosis with systolic dysfunction (Hrynchyshyna et al. 2013). However, galectin-3 concentration is not correlated with echocardiographic and hemodynamic HF data (Hrynchyshyna et al. 2013).

Elimination from the Circulation Issues

Another potential confounding factor in the interpretation of blood concentrations of collagen markers is their pathway of elimination. The propeptides PICP and PIIINP are cleared via uptake by endothelial cells in the liver (Lopez et al. 2010; Smedsrød et al. 1990; Melkko et al. 1994). As a consequence, in conditions of chronic liver insufficiency, the increases in their serum concentrations may reflect reduced hepatic clearance but not increased production (Lopez et al. 2010). On the other hand, ICTP is believed to be cleared through the kidneys because of its small size (12 kDa)

(Lopez et al. 2010). Thus, a glomerular filtration rate <50 mL/min/1.73 m^2 facilitates the increase in ICTP serum concentration (Lopez et al. 2010; Risteli et al. 1993).

An inverse relationship between galectin-3 and renal function has been observed in patients with HF (Shah et al. 2010; Lok et al. 2010; Tang et al. 2011; Gopal et al. 2012). There are several potential mechanisms for increased galectin-3 levels in the setting of renal impairment. First, it is possible that galectin-3 (molecular weight [asymptotically equal to] 30 kDa) clearance is via the kidney and thus serves primarily as a marker of reduced renal function. Second, there could be increased production of galectin-3 by the kidney. Third, it is possible that galectin-3 is produced in the kidney and exerts profibrotic effects resulting in renal function impairment (Gopal et al. 2012).

Demographic Issues

Several demographical variables have been suggested to influence collagen peptide levels. Age, gender, body mass index, level of exercise, smoking, and fasting status are some among many that have been reported to confound circulating levels of ECM-related biomarkers (Lopez et al. 2010; De Jong et al. 2011; Chubb 2012). For example, it has been reported that PIIINP levels increased with age and body mass index (Wang et al. 2007). This implies that in further investigations, cardiac remodeling biomarker levels should be adjusted for several patient characteristics, at least for body surface area, gender, and age (De Jong et al. 2011). Biologic variation is also an additional potential confounding factor. It has been shown that either circadian or long-term biologic variation for some of these collagen peptides may reach to levels of up to 30 % (Chubb 2012).

For galectin-3 levels, a recent study showed that it is characterized by a lower biological variation than the natriuretic peptides (Wu et al. 2013). However, several clinical studies have identified that galectin-3 concentration is probably correlated with age (Hrynchyshyna et al. 2013).

Analytical Issues

In general, biomarkers with high within-individual or between-individual biological variation are less clinically useful than those that have tight limits (Wu 2013). Most studies on biological variation are conducted on an appropriate population of healthy subjects. It is essential to understand the variances in health before test results can be applied to diseased patients (Wu 2013). The utility of a marker depends on its sensitivity and ability to discriminate a normal value from an abnormal value. The sensitivity and discriminatory values are, in turn, a function of the stability of these markers over time, which can be assessed by within-subject variability and analytic imprecision (Nguyen et al. 2008). Conceptually, the variation of a biochemical marker can be partitioned into two major sources, between-subject and within-subject

Table 8 Estimates of variance components due to within-subject and between-subject sources

Marker	Coefficient of reliability[a]	Index of individuality	Between-subject variance (% total variability)	Within-subject variance		
				Total within-subject (% total variability)	Biological variance (% within-subject variance)	Analytic variance (% within-subject variance)
PINP	0.96	0.20	96.2	3.8	92.8	7.2
ICTP	0.91	0.32	90.0	10.0	95	5.0
PIIINP	0.87	0.38	87.2	12.8	90.9	9.1

Index of individuality is calculated as the ratio of the biologic standard deviation to the between-subject standard deviation. The index indicates the degree to which a single measurement in this population is able to distinguish unusual results in a subject (Nguyen et al. 2008)

Adapted from Nguyen et al. (2008), *ICTP* collagen type I cross-linked carboxy-terminal telopeptide, *PICP* C-terminal propeptide of collagen type 1, *PINP* N-terminal propeptide of collagen type 1, *PIIINP* N-terminal propeptide of collagen type III

[a]Coefficient of reliability is defined as the ratio of the between-subject variance to the total variance (the sum of between- and within-subject variances), for each marker. This coefficient can be viewed as the degree to which individuals' marker values remain relatively consistent over repeated measurements (Nguyen et al. 2008)

variation, and the latter further partitioned into normal biological variation and analytical (random) imprecision of the method of measurement (Nguyen et al. 2008).

In a recent study, the short-term within-subject variability of collagen markers (PINP, ICTP, and PIIINP) was determined in a large cohort of 1,000 elite athletes. The within-subject variability was partitioned into biologic and analytic variability and was compared to the between-subject variability to assess the implications of this variability in the clinical setting (Nguyen et al. 2008). Regarding markers' laboratory measurements, competitive radioimmunoassays were used (Orion Diagnostica) in the same assay batch with the following intra- and interassay coefficient of variation both <10 % for ICTP; 9 % and 12 %, respectively, for PINP; and 7 % and 12 %, respectively, for PIIINP (Nguyen et al. 2008).

In Table 8, the between-subject and within-subject biological variation of the aforementioned markers are reported. Between-subject variance formed a major component of the variance of the markers, accounting for 87–96 % of total variance of collagen markers (Nguyen et al. 2008). Thus, within-subject variance accounted for between 4 % and 13 % of the total variance of the markers (Nguyen et al. 2008). Most of the within-subject variance was attributable to biological variability (90–95 % of the within-subject variance) (Nguyen et al. 2008). The within-subject variability was subsequently transformed into the original unit of measurement to yield the standard deviation (SD) and then expressed as the coefficient variation (CV) relative to the mean (Nguyen et al. 2008). The short-term within-subject SD ranged from 13 % to 15 % for the three bone turnover markers and the CV due to analytic variability ranged from 3 % to 4.5 % was smaller than the CV for biological variability (ranged from 12.8 % to 14.2 %) (Nguyen et al. 2008). In another study

reporting on variability of PICP, the CV due to analytical variability was 5.1 % and the corresponding CV for biological variability was 7.8 % (Hannon et al. 1998). The coefficient of reliability was high for the collagen markers (R 0.87–0.96) indicating that the within-subject variability was smaller relative to between-subject variability (Nguyen et al. 2008). The index of individuality for all collagen markers was <0.6, indicating that although these markers produce stable results within an individual over time, the population-based reference range is of limited value for the interpretation of measurements in an individual, because test results that are abnormal for the individual can be undetected by the population-based reference range (Nguyen et al. 2008). Thus, a single value cannot be compared against a population range, and serial testing would be necessary for interpretation (Wu 2013).

Regarding galectin-3 levels, a recent study reported that the between-subject CV ranged from 16 % to 23 %, and the within-subject CV ranged from 16 % to 20 %. The analytical variation (CVa) was 10.6 % or 39 % of the biological variation (Wu et al. 2013). The index of individuality was approximately 1.0, and the coefficient of reliability was 0.52 (Wu et al. 2013).

Taken all of the above into consideration, collagen markers have increased between-subject variability and low index of individuality rendering difficult to construct population reference levels. On the other hand, the smaller within-subject variability in conjunction with high coefficient of reliability and low index of individuality renders collagen markers ideal for serial testing and monitoring progression of the underlying disease. Finally, the low analytic variability (<5 %) suggests that assessment of their concentration is reliable at least with radioimmunoassay. In contrast, for galectin-3 levels, the high individuality index suggests that it would be appropriate to compare results on a given patient against values from a reference population.

Comorbidity Issues

Because fibrosis also occurs in other organs, it is possible that the increased levels of these biomarkers are not only from cardiac origin but also from other diseased organs such as the bone, liver, kidneys, and lungs (De Jong et al. 2011; Lijnen et al. 2012). Some published data suggest that changes in concentrations of some of these biomarkers present in patients with vascular diseases represent integrated abnormalities of the cardiovascular collagen (Lopez et al. 2006; Chatzikyriakou et al. 2008; McNulty et al. 2006). These observations suggest that in arterial hypertension (Lopez et al. 2006; McNulty et al. 2006) or heart failure (Lopez et al. 2006; Chatzikyriakou et al. 2008), alterations in these molecules may reflect disturbances of collagen metabolism that occur not just at the myocardial but also at the arterial wall level.

In addition, the presence of concomitant non-cardiovascular diseases affecting collagen matrix can also affect the circulating levels of these molecules because none of them are exclusively from a cardiac origin (Table 9) (Lopez et al. 2010; Demers et al. 2000; Christenson 1997; Grigorescu 2006; Urena and de Vernejoul 1999; Nakamura 2000; Thickett et al. 2001). Therefore, as these peptides cannot be specifically attributed to alterations in myocardial collagen network, before using

Table 9 Circulating collagen metabolism biomarkers in patients with non-cardiac diseases

Non-cardiac diseases	Collagen metabolism biomarker
Metastatic bone disease (breast, lung, or prostate cancer)	ICTP
Metabolic bone disease (severe osteoporosis)	PICP, PINP
Chronic liver diseases (cirrhosis of different etiologies)	PIIINP, galectin-3
Chronic kidney disease (stages 4 and 5 with high-turnover bone disease)	PICP, ICTP, galectin-3
Other inflammatory and fibrotic diseases	
Osteoarthritis, rheumatoid arthritis	PIIINP, ICTP
Diffuse fibrotic lung disease	PICP, ICTP, galectin-3
Chronic pancreatitis	Galectin-3

Adapted from Chalikias and Tziakas (2014) by permission; Elsevier License number 3538261127514; Lopez et al. 2010), *ICTP* collagen type I cross-linked carboxy-terminal telopeptide, *PICP* C-terminal propeptide of collagen type 1, *PINP* N-terminal propeptide of collagen type 1, *PIIINP* N-terminal propeptide of collagen type III

them as circulating biomarkers of collagen metabolism, the presence of these conditions must be excluded in the patients under study (Lopez et al. 2010).

Regarding galectin concentrations, upregulation of galectin-3 has been demonstrated in different human fibrotic conditions, such as in liver cirrhosis, idiopathic lung fibrosis, and chronic pancreatitis (de Boer et al. 2010). Furthermore, increased galectin-3 expression has been shown in renal injury or failure especially during renal fibrosis (Fernandes et al. 2008; Nishiyama et al. 2000) (Table 9).

Pharmacological Treatment Issues

Cardiovascular drugs and standard therapies used in cardiovascular diseases may modify circulating levels of collagen metabolism-related biomarkers as it has been previously shown (effect of standard therapies) (Lopez et al. 2010; De Jong et al. 2011). It has been reported that serum or plasma levels of these peptides may change in patients being treated with angiotensin-converting enzyme inhibitors (Tziakas et al. 2012; Chatzikyriakou et al. 2010), angiotensin type 1 receptor antagonists (Diez et al. 2002; Lopez et al. 2005; Ciulla et al. 2004; Muller-Brunotte et al. 2007), diuretics (Lopez et al. 2004), or aldosterone antagonists (Zannad et al. 2000; Deswal et al. 2011; Udelson et al. 2010; Iraqi et al. 2009; Li et al. 2009; Mak et al. 2009). Furthermore, other drugs used in cardiovascular conditions different to hypertension or heart failure, such as statins, have been shown to modify ECM-related biomarker levels (Rejnmark et al. 2002). Moreover, drugs may influence biomarker levels indirectly, for example, via the elimination of the peptides by the liver (De Jong et al. 2011). Thus, the potential influence of previous pharmacological treatment must be carefully considered when assessing serum biomarkers of collagen metabolism in cardiovascular patients (Lopez et al. 2010).

Concluding Remarks

Despite the available information on circulating biomarkers of collagen metabolism, a number of major limitations as shown above remain that weaken their clinical usefulness (Lopez et al. 2010). Unfortunately, based on current knowledge, it is not possible to determine prognostic, diagnostic, or treatment management cutoff values for different cardiomyopathies (De Jong et al. 2011). Future research initiatives aimed at the following aspects are urgently needed:

1. Demonstration of the association between the measured levels of these biomarkers, the myocardial collagen network, and the alterations of left ventricular anatomy and function
2. Prospective validation of incremental information provided by a multimarker strategy combining these peptides with standard biochemical markers (for instance, natriuretic peptides or troponins)
3. Assessment of effects of their measurements on patient management and outcomes
4. Evaluation of the feasibility and cost-effectiveness of the application of this strategy in the community
5. Standardization of the laboratory methods used to measure them

Summary Points

- Extracellular matrix alterations are present in all spectrum of cardiovascular diseases and especially heart failure.
- Collagen, types I and III, is the principal structural protein found in the myocardium and its pro- or telopeptides are released into the circulation during collagen synthesis and degradation.
- These collagen-derived pro- and telopeptides may reflect collagen synthesis and breakdown and also represent a much more useful tool to address extracellular matrix changes from a distance.
- Galectin-3 signaling results in fibroblast and myofibroblast proliferation and synthesis of procollagen type I.
- Galectin-3 may reflect myocardial fibrosis.
- Despite being used as circulating biomarkers of collagen metabolism in numerous clinical studies in heart failure, a number of major limitations remain that weaken their clinical usefulness.

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PCSK9 as a Biomarker of Cardiovascular Disease

6

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_20

Abstract

PCSK9 is a secreted protein which in circulation promotes lysosomal degradation of the low-density lipoprotein receptor (LDLR). Hence, it is informative to measure its concentration in blood. Circulating PCSK9 exists in several forms – its parent form and a furin-cleaved form and a low-density lipoprotein (LDL)-bound form and a free form. The furin-cleaved and LDL-bound forms are much less active. Most studies have not clearly distinguished among these forms. In a different context, in individuals receiving anti-PCSK9 monoclonal antibody (mAb) therapy, there is an Ab-bound form and a mAb-free form. Future measurements of circulating PCSK9 will need to account for the heterogeneity of PCSK9 in circulation.

Circulating PCSK9 concentration shows a diurnal rhythm and it decreases beyond ~16 h of fasting. It has a wide 50- to 100-fold range within a population and is rightward skewed, higher in women than men, and higher after menopause. In male adolescents, it decreases with age, while in female adolescents, it increases with age. It is elevated in pregnancy at term, and umbilical cord blood has lower serum PCSK9 levels than maternal blood. Ideally, measurement of plasma PCSK9 should be standardized to time of day and feeding status.

Plasma PCSK9 levels associate with LDL cholesterol (LDL-C) levels in most states of health and disease. The positive correlation between serum PCSK9 and LDL-C is relatively weak in contrast to the much greater effect of genetic variation in PCSK9 activity on serum LDL-C levels. GOF and LOF mutations and polymorphisms of PCSK9 are often reflected in higher and lower plasma PCSK9 levels, respectively, but this is not always the case.

Plasma PCSK9 measurement in various states of health and disease has enhanced our knowledge of lipoprotein metabolism in general and in abnormal states associated with CVD. It is currently not seen as a clinical marker of CVD risk or disease progression. There are early reports indicating an association between plasma PCSK9 levels and vascular disease, independent of other traditional risk factors, but underlying pathophysiologic mechanisms are still unclear. Plasma PCSK9 may serve in the future as a biomarker for the selection of patients for anti-PCSK9 mAb therapy.

It has also been measured in response to various forms of lipid-altering drug therapy. Statin therapy increases plasma PCSK9 levels. These data will need further development in order to serve as an aid to the establishment of the pharmacokinetic and pharmacodynamic properties of various forms of therapy. In clinical practice, it could be helpful in determining statin dosage, but its measurement is not essential as it is possible to simply titrate the dose of statin upward or downward as guided by LDL-C response. In clinical research, plasma PCSK9 levels provide hypothesis-generating information which could help the design of basic science experiments that explore mechanisms of regulation, metabolism, and role of PCSK9.

Keywords

PCSK9 • Circulating forms • Biomarker • Cardiovascular disease • Health and disease • Statins

Abbreviations

ApoB100	Apolipoprotein B100
BMI	Body mass index
CAD	Coronary artery disease
cIMT	Carotid intima-media thickness
CKD	Chronic kidney disease
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CV	Cardiovascular
CVD	Cardiovascular disease
EGF-A	Epidermal growth factor-like repeat A
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FH	Familial hypercholesterolemia
FIELD	Fenofibrate intervention and event lowering in diabetes
GOF	Gain of function
HeFH	Heterozygous familial hypercholesterolemia
HoFH	Homozygous familial hypercholesterolemia
LDL	Low-density lipoprotein
LDL-A	Low-density lipoprotein apheresis
LDL-C	Low-density lipoprotein cholesterol
LDLR	Low-density lipoprotein receptor
LOF	Loss of function
mAb	Monoclonal antibody
MS	Mass spectrometry
NARC-1	Neural apoptosis-regulated convertase 1
Non-FH	Nonfamilial hypercholesterolemia
PCSK9	Proprotein convertase subtilisin-kexin 9
SNP	Single-nucleotide polymorphism
SREBP2	Sterol-responsive element-binding protein 2
T1DM	Type 1 diabetes mellitus
TC	Total cholesterol
T2DM	Type 2 diabetes mellitus
TG	Triglycerides
TRL	Triglyceride-rich lipoproteins
TSH	Thyroid-stimulating hormone
VLDLR	Very low-density lipoprotein receptor

Key Facts of PCSK9

- PCSK9 was first discovered in 2003 as an important regulator of blood cholesterol levels through the identification of individuals with PCSK9 mutations that led to more active forms of PCSK9 and very high blood cholesterol levels.
- Conversely, mutations of the PCSK9 gene that lead to less active forms of PCSK9 are associated with lower blood cholesterol levels and lower risk for coronary artery disease.
- Drugs that inhibit PCSK9 action are already under development to treat high blood cholesterol.
- Since PCSK9 is active mostly while it is in blood circulation, it is informative to measure its level in blood.
- Measurement of blood levels of PCSK9 has provided added information on how cholesterol and other blood fats are regulated in the body in health and in various disease conditions.
- Blood PCSK9 levels are also directly linked to disorders that result from blockages in blood vessels, independent of other risk factors such as blood cholesterol levels.
- Treatment of high blood cholesterol with statins, a very effective and widely used class of cholesterol-lowering drugs, increases plasma PCSK9 levels, thereby blunting their full cholesterol-lowering effect.
- The addition of anti-PCSK9 therapy has the ability to further lower blood cholesterol levels in those who are already on statins.

Definitions

Carotid intima-media thickness A measurement of the inner two layers of the main artery in the neck to evaluate the presence, progression, or regression of atherosclerosis

Gain-of-function mutation A mutation that results in an enhanced activity of the protein encoded by the gene

Heterozygote An individual with a gene variation that affects a single allele of a specific gene

Homozygote An individual with a gene variation that affects both alleles of a specific gene

Loss-of-function mutation A mutation that results in reduced or no activity of the protein encoded by the gene

Mutation A change in the nucleotide sequence of a gene that is rare (usually <1 % of the population) and may be associated with a clear manifestation of disease

Parabiosis An intracellular enzyme that converts a variety of inactive precursor proteins within cells into active products

Single-nucleotide polymorphism A variation of a single nucleotide in the nucleotide sequence of a gene. This is often seen in a significant proportion of the population and is more common than a mutation. Its effect on biologic function is usually less than that of a mutation

Sterol-responsive element-binding protein 2 A protein that upregulates synthesis of enzymes involved in intracellular sterol production

Variant/variation Any change in the nucleotide sequence of a gene. This term includes mutations and polymorphisms

Introduction

Proprotein convertase subtilisin-kexin 9 (PCSK9) was discovered in 2003 as the ninth member of the secretory subtilase family of proprotein convertases (PCs) that are responsible for proteolytic activation of precursor proteins in the secretory pathway. PCSK9 is expressed primarily in the liver, small intestine, kidneys, and brain (Seidah et al. 2003). It was initially named neural apoptosis-regulated convertase 1 (NARC-1) for its ability to enhance recruitment of undifferentiated neural progenitor cells into the neuronal lineage (Seidah et al. 2003). The importance of PCSK9 in lipoprotein homeostasis was recognized in the same year, when two single-nucleotide polymorphisms (SNPs) in the *PCSK9* gene resulting in the amino acid conversion of S127R within the prodomain and F216L within the catalytic domain of PCSK9 were shown to associate with a form of autosomal dominant familial hypercholesterolemia (FH) (Abifadel et al. 2003). Conversely, loss-of-function (LOF) variants of PCSK9 were subsequently shown to be associated with lower low-density lipoprotein cholesterol (LDL-C) and a significant reduction in risk of coronary artery disease (CAD) (88 % and 47 % for C679X and R46L heterozygotes, respectively) (Cohen et al. 2006).

These clinical observations were accompanied by overexpression studies of PCSK9 in mice which showed a reduction in LDLR protein but not mRNA, suggesting that PCSK9 accelerated turnover of the LDLR, resulting in hypercholesterolemia (Benjannet et al. 2004; Maxwell and Breslow 2004; Park et al. 2004). On the other hand, knockout of *PCSK9* in mice resulted in a 2.8-fold increase in LDLR protein (compared to wild-type littermates) leading to increased clearance of LDL and a decrease in plasma cholesterol levels of about 40 % (Rashid et al. 2005). Again, mRNA was not affected. PCSK9 mRNA levels are responsive to cellular cholesterol levels through the transcription factor, sterol-responsive element-binding protein 2 (SREBP2) (Horton et al. 2003; Maxwell et al. 2003).

Subsequent studies showed that secreted PCSK9 binds to the epidermal growth factor-like repeat A (EGF-A) domain of the LDLR (Zhang et al. 2007) and is

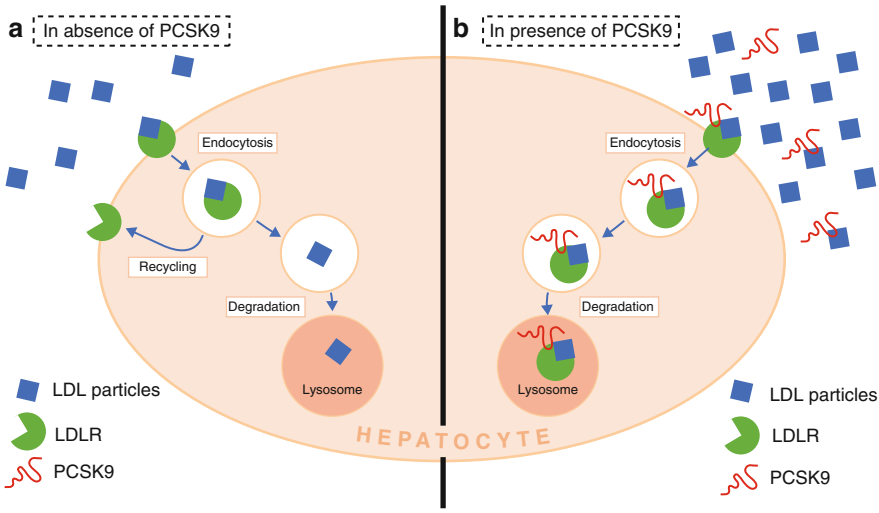

Fig. 1 Mechanism of action of PCSK9. (**a**) In the absence of PCSK9, the LDL particle in the endosome is directed to the lysosomal compartment for degradation, while the LDLR is recycled back to the cell surface through the endosomal recycling pathway. (**b**) In the presence of PCSK9, PCSK9, bound to the EGF-A domain of the LDLR, is endocytosed with the LDL-LDLR complex. PCSK9 prevents recycling of LDLR to the cell surface and directs LDL-LDLR to the lysosomal compartment for degradation

endocytosed with the LDLR (Lagace et al. 2006). PCSK9 redirects the LDLR from the endosomal recycling pathway to the lysosomal compartment for degradation, thereby modifying circulating LDL-C levels (Fig. 1) (Nassoury et al. 2007; Zhang et al. 2007).

There is significant evidence that PCSK9 is secreted before it interacts with cell surface LDLR. The introduction of PCSK9 into mice, directly or through parabiosis, reduced hepatic LDLR levels (Lagace et al. 2006). Addition of recombinant PCSK9 to medium of cultured cells results in LDLR degradation (Lagace et al. 2006; Fisher et al. 2007). This need for secretion draws attention to the potential value of measuring the concentration of PCSK9 in circulation. There is also evidence that PCSK9 may interact with the LDLR without prior secretion (Poirier et al. 2009).

Although it is identified as a member of a family of proteolytic enzymes, PCSK9's natural substrate is unknown and so is its physiological function, although its promotion of LDLR degradation indicates a role in cholesterol homeostasis. It is interesting to note that this role does not require its enzymatic property (McNutt et al. 2007).

Detection of PCSK9 in Human Circulation

The initial experiments on NARC-1 clearly showed that CHO and HK293 cells, stably or transiently transfected with human or mouse *NARC-1*, secreted NARC-1/PCSK9 into the culture medium (Seidah et al. 2003). This observation suggested

that PCSK9 could be detected in human plasma. Confirmation of this was soon published (Alborn et al. 2007; Mayne et al. 2007, 2008; Lambert et al. 2008; Dubuc et al. 2010). In studies involving total and liver-specific *pcsk9* knockout mice, it has been demonstrated that the main source of circulating PCSK9 is the liver, despite significant expression in the intestine and kidneys (Rashid et al. 2005; Zaid et al. 2008).

PCSK9 has since been measured in plasma or serum in normal and abnormal conditions, and this chapter reviews the potential role of circulating PCSK9 measurement as a biomarker in cardiovascular disease (CVD). A biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Biomarkers Definitions Working Group 2001). This chapter examines PCSK9's role as an indicator of "normal biologic processes," "pathogenic processes," and "pharmacologic responses to a therapeutic intervention," but it will be restricted to the context of CVD. Before doing this, we discuss the various forms of circulating PCSK9 and address the question of whether plasma PCSK9 levels reflect genetic variance of the *PCSK9* gene.

Circulating Forms of PCSK9

PCSK9 is synthesized as a ~74 kDa proprotein from which a prodomain of ~14 kDa is autocatalytically cleaved within the secretory pathway. The prodomain remains attached to PCSK9 by non-covalent bonds. This autocatalytic cleavage is essential for the exit of PCSK9 from the endoplasmic reticulum (ER) as demonstrated in the Q152H variant in which this cleavage fails to occur resulting in markedly lowered plasma PCSK9 levels (Mayne et al. 2011). There is also evidence that in the absence of the N-terminal sequence of the prodomain (aa 31–58), the PCSK9 complexed with the prodomain is severalfold more active in degrading LDLR, suggesting that the prodomain is a negative regulator of PCSK9's degradation activity on the LDLR (Benjannet et al. 2010). It is likely that most reported assays of PCSK9 measure the prodomain-associated PCSK9 molecule.

There are three other issues about circulating PCSK9 to address. The various forms of circulating PCSK9 are illustrated in Fig. 2. PCSK9 circulates as its parent form as well as a furin-cleaved form. Membrane-bound furin, another PC, cleaves the parent PCSK9 at the Arg^{218}-Gln^{219} peptide bond (Benjannet et al. 2006; Essalmani et al. 2011) to result in a furin-cleaved form that lacks a ~7 kDa segment, Ser^{153}-$Arg^{218,}$ within the catalytic domain. The ~53 kDa furin-cleaved PCSK9 has been reported to have no LDLR degradation activity likely because of the loss of a region of PCSK9 required for LDLR binding as well as the concomitant loss of the ~14 kDa prodomain (Benjannet et al. 2006; Essalmani et al. 2011). Another study, however, has shown that furin cleavage did not result in dissociation of the prodomain (Han et al. 2014). With regard to LDLR degradation activity, yet another study has shown that furin-cleaved PCSK9 does have LDLR degradation activity, but it is less efficient than the intact parent PCSK9 (Lipari et al. 2012).

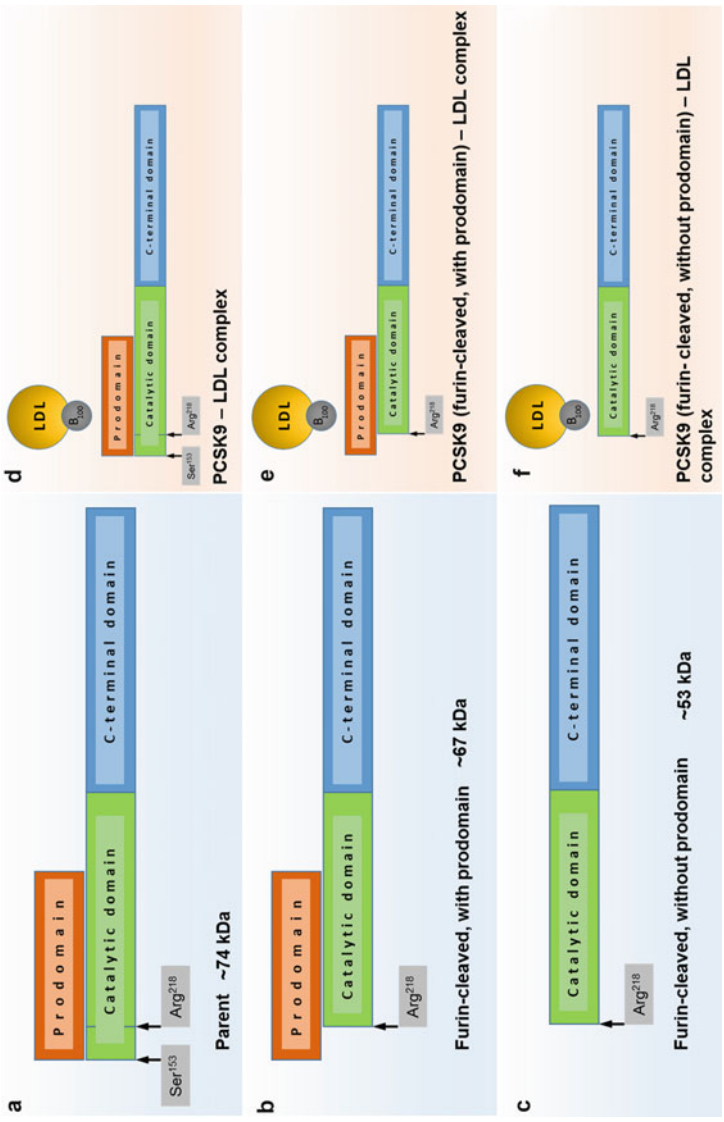

Fig. 2 (continued)

Until recently, most reports on circulating PCSK9 have not distinguished between the parent PCSK9 and furin-cleaved PCSK9. However, two recent reports have distinguished between these two forms of PCSK9 (Han et al. 2014; Hori et al. 2015). A method to isolate the truncated form from the parent PCSK9 was developed using differential binding of monoclonal antibodies (mAbs) to the two forms; the C-terminal domain PCSK9 mAb binds both forms and a catalytic domain mAb binds only the parent PCSK9 (Han et al. 2014). Mass spectrometry (MS) and ELISA methods to detect PCSK9 indicated that ~30 % of total PCSK9 was in the truncated furin-cleaved form. This was demonstrated in serum samples from only 20 human subjects. A recombinant form of this truncated PCSK9 was shown to be inactive on LDLR. They further showed that the weak correlation demonstrated between total PCSK9 (parent plus furin-cleaved PCSK9) and LDL-C was not improved by considering only the active parent form of PCSK9 (Han et al. 2014). Confirmation of these findings in a larger population is needed.

The other group that looked into this also used differential binding of mAb to measure the two forms of circulating PCSK9 (Hori et al. 2015). They found that the furin-cleaved form constituted 15 % of total PCSK9, unlike the study of Han et al. in which the figure was 30 %. They also demonstrated that both forms of PCSK9 was removed by LDL apheresis (LDL-A) by about 46–56 %.

Apart from these two studies, most other studies reporting on serum or plasma PCSK9 levels have not distinguished between the parent and the furin-cleaved forms of PCSK9. Since the furin-cleaved form has been shown to be inactive or less active, it is theoretically necessary to measure the parent form alone for proper interpretation of the role of PCSK9 activity in any situation. However, whether it is essential to do so is still unclear. A superior method that could distinguish the various forms of circulating PCSK9 is MS (Dewpura and Mayne 2011), but this is expensive and not widely available.

The next aspect of circulating PCSK9 is that ~30–40 % is associated with LDL particles via an interaction with apolipoprotein B100 (apoB100) (Sun et al. 2012; Kosenko et al. 2013; Tavori et al. 2013), likely at a single apoB100 molecular site (Kosenko et al. 2013). In one study, PCSK9 associated with LDL is a monomer, while the rest of unassociated PCSK9 is found in larger complexes. It also showed that PCSK9 existing as complexes is the active form of PCSK9 (Fan et al. 2008; Kosenko et al. 2013). However, there is also evidence that monomeric PCSK9 is active (Lagace et al. 2006). In cell culture studies, LDL inhibited PCSK9 binding to

Fig. 2 Multiple forms of circulating PCSK9. The parent form of PCSK9 has a prodomain that is attached to it by non-covalent bonds. (**a**) About 15–30 % of PCSK9 has a ~7 kDa segment, Ser153–Arg218, within the catalytic domain cleaved off by furin. The prodomain has been variably reported as either remaining attached (**b**) or concomitantly lost from the parent molecule upon furin cleavage (**c**). About 30–40 % of these intact (**d**) and furin-cleaved PCSK9, in turn, are bound to LDL particles via an interaction with apoB100 (**e**, **f**). The binding sites on PCSK9 and apoB100 have not been clearly determined. Not shown in the figure are PCSK9 molecules that are bound to monoclonal antibodies (mAbs) in patients receiving anti-PCSK9 mAb therapy for treatment of hypercholesterolemia

LDLR, and this was independent of the LDL-LDLR interaction (Kosenko et al. 2013). Specifically in FH patients, gel filtration chromatography analysis has shown that 20 % of total plasma PCSK9 exists in the apoB-containing fraction, and both the parent and the furin-cleaved forms of PCSK9 in this fraction were reduced by 92–97 % by LDL-A.

Another aspect to consider about measurement of circulating PCSK9 is that during anti-PCSK9 mAb therapy, papers have reported on measurement of plasma-free or unbound PCSK9 levels. In this setting, free PCSK9 refers to PCSK9 that is unbound to the mAb. It is unclear whether the unbound PCSK9 is partially bound to LDL particles and whether it is total or only mature or only furin-cleaved PCSK9 that is measured. The method for measurement of free PCSK9 in evolocumab trials has been well characterized (Colbert et al. 2014). Plasma-free PCSK9 levels were decreased by as much as ~90 %, the magnitude and duration of PCSK9 reductions being related to the extent and duration of LDL-C reductions (Blom et al. 2014). In trials on alirocumab, both total and free PCSK9 levels have been measured. Total PCSK9 was obtained by dissociating soluble PCSK9 from PCSK9/alirocumab complexes by means of an acid-wash step. In these trials, plasma total PCSK9 levels were markedly increased, likely the result of slower clearance of the bound complex from the circulation. In contrast, there was a marked reduction in free PCSK9 concentration (Roth et al. 2012). These are not unexpected findings. Plasma-free PCSK9 levels have the potential of serving as a biomarker of pharmacological response. However, the full value of plasma total and free PCSK9 measurement as an aid to the establishment of the pharmacokinetic and pharmacodynamic properties of this form of therapy has not been fully developed (Colbert et al. 2014).

PCSK9 Is a Polymorphic Gene: Do Plasma PCSK9 Levels Reflect Gene Variation?

PCSK9 is located on human chromosome 1(1p32) and has 12 exons. Many studies have demonstrated the presence of a large number of variations in the *PCSK9* gene (Abifadel et al. 2009). Among these variants, some are gain-of-function (GOF) mutations that are associated with a clear phenotype such as autosomal dominant hypercholesterolemia, while others are LOF mutations associated with hypocholesterolemia. In addition to these important but rare mutations, polymorphisms that have smaller effects (some elevating, others lowering) or no effects on LDL-C levels are much more common.

The key question here is whether the GOF or LOF is mediated through an increase or a decrease in circulating plasma PCSK9 levels, respectively. This would be dependent on the functional defects associated with the mutation/polymorphism. For those that are associated with decreased secretion of PCSK9 due to various secretory defects, LOF would be expected to be mediated by decreased circulating PCSK9 levels. There is a report of a young female with compound heterozygosity for LOF PCSK9 mutations who had both a very low LDL-C and

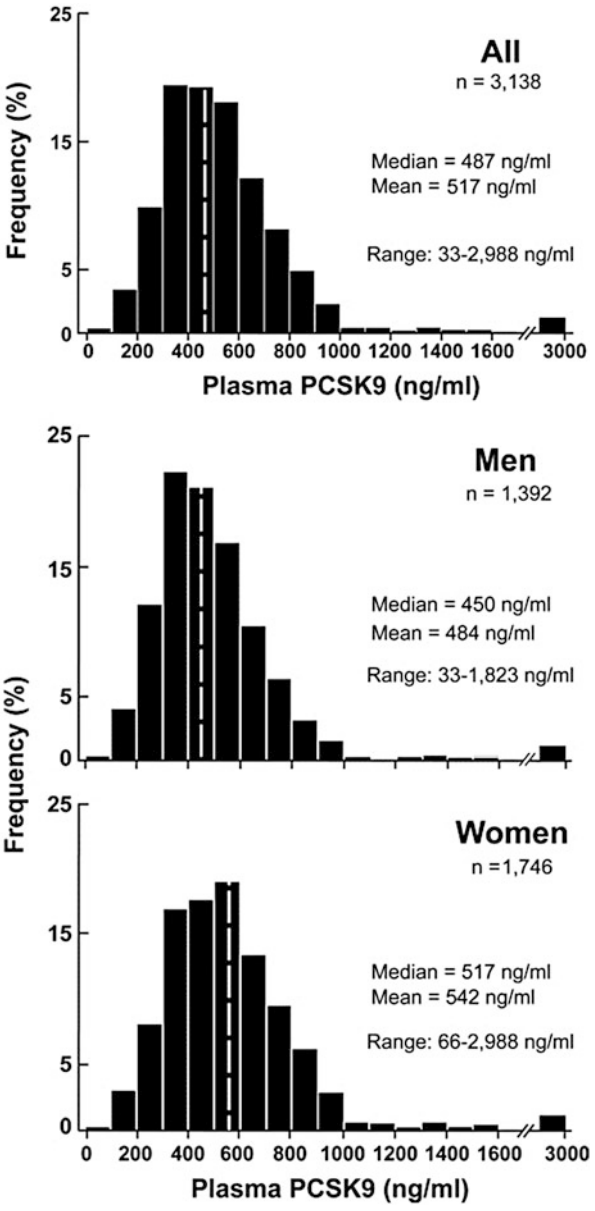

Fig. 3 Distribution of fasting plasma concentrations of PCSK9 in the Dallas Heart Study. The distribution of fasting plasma concentrations of PCSK9 in the Dallas Heart Study ($n = 3138$) excluding individuals on statins (97 women and 117 men) in all subjects (**a**), in men only (**b**; $n = 1392$), and in women only (**c**; $n = 1746$). Significant differences were observed between men and women after adjustment for age, ethnicity, BMI, diabetes, and plasma levels of LDL-C, HDL-C, and triglycerides ($P < 0.0001$) (Reprinted with permission from Lakoski et al. 2009)

an undetectable level of plasma PCSK9 (Zhao et al. 2006). However, the functional defects associated with mutations/polymorphisms are not all known.

In the Dallas Heart Study where plasma PCSK9 levels were measured in individuals with known mutations/polymorphisms, it was shown that LOF mutations/polymorphisms were associated with lower plasma PCSK9 levels (Fig. 3) (Lakoski et al. 2009). However, there are exceptions to an association between

LDL-C effect and circulating PCSK9 levels. Subjects with the GOF variant, D374Y, had markedly elevated LDL-C, but their plasma PCSK9 levels were low (Humphries et al. 2009). In subjects with the minor A53V variant which is associated with a reduction in LDL-C by ~15 %, there was no difference in their plasma PCSK9 levels from normal controls with no *PCSK9* variant (Mayne et al. 2013).

Given the large body of information available on PCSK9's role in LDL clearance, variants are currently designated as having GOF or LOF properties based on their effect on serum LDL-C levels. As knowledge of other possible functions of PCSK9 evolve, it is possible that the definition of GOF and LOF pertaining to PCSK9 may change. This concept is particularly plausible in the area of triglyceride-rich lipoprotein metabolism (Tavori et al. 2015).

Physiological Status of Circulating PCSK9

Circulating PCSK9 shows a diurnal rhythm synchronous with cholesterol synthesis and growth hormone secretion. It also shows a significant fall of ~35 % after 18 h of fasting (Persson et al. 2010). In another study, blood was collected every 4 h from the start of fasting and there was no change in serum PCSK9 level after 12 h of fasting, but a significant fall of ~14 % was seen at 16 h (Browning and Horton 2010). Ideally, measurement of plasma PCSK9 should be standardized to time of day and feeding status.

Diet also seems to have an effect on circulating plasma PCSK9 levels. A Mediterranean diet that reduces LDL-C is associated with a reduction in PCSK9 levels even in the absence of weight loss (Richard et al. 2012).

PCSK9 concentrations vary very widely among studies, likely the result of different binding characteristics of various polyclonal and monoclonal antibodies and PCSK9 standards used in the assays. Thus, inter-study comparisons of absolute PCSK9 levels should not be made until standardized assays are used. Plasma PCSK9 concentrations also show a wide range within a population using a single assay. It was measured by ELISA in the large multiethnic population of the Dallas Heart Study ($n = 3138$) (Lakoski et al. 2009). The distribution of fasting plasma PCSK9 levels in this population is shown in Fig. 4. They showed a rightward skew in distribution of PCSK9 levels in the population. There was a 100-fold range in plasma PCSK9 levels. Females had higher levels than males, and this difference persisted after adjusting for age, ethnicity, BMI, systolic BP, menopausal status, and fasting levels of glucose, LDL-C, HDL-C, TG, and CRP. Postmenopausal women had higher levels than premenopausal women. Curiously, in postmenopausal women, there was no difference in PCSK9 levels between those receiving and those not receiving estrogen replacement therapy, suggesting that the higher PCSK9 levels in postmenopausal women may be the result of diminished LDLR-mediated clearance of PCSK9 since hypoestrogenemia in menopause is associated with a decrease in LDLR (Walsh et al. 1991).

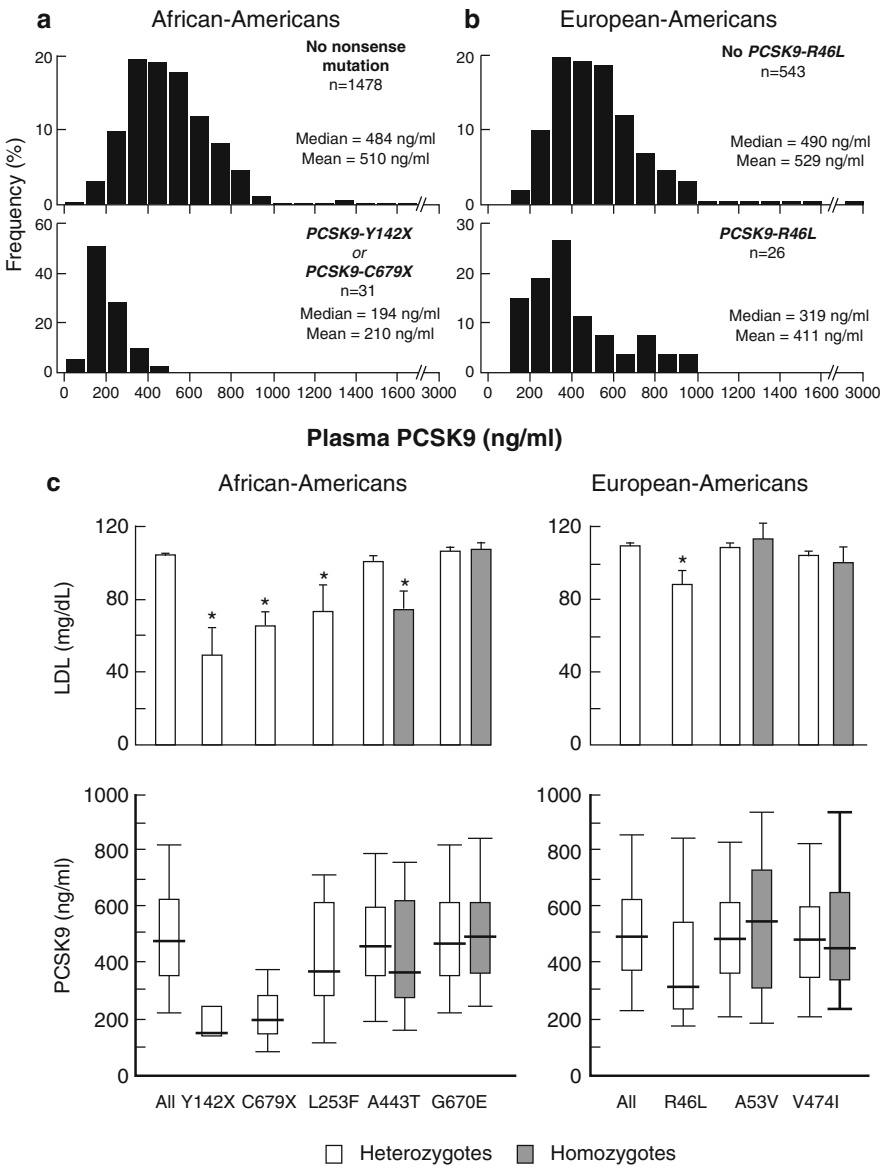

Fig. 4 Distribution and median plasma levels of plasma PCSK9 levels in African-Americans and European-Americans with various sequence variations in PCSK9. The distribution of plasma PCSK9 levels in African-Americans who were heterozygous for a nonsense mutation in PCSK9 (Y142X or C679X) (**a**), European-Americans heterozygous or homozygous for PCSK9:R46L (**b**), and median plasma levels of PCSK9 in African-Americans and European-Americans with various sequence variations in PCSK9 (**c**). (**a**) African-American individuals heterozygous for a nonsense mutation had significantly lower median PCSK9 concentrations compared with those without the mutation after adjusting for age and sex ($P < 0.0001$) and after adjusting for LDL-C ($P < 0.0001$). (**b**) In European-Americans, individuals heterozygous for R46L had significantly lower plasma

In a population of 1739 French-Canadian children and adolescents aged 9, 13, and 16 years, plasma PCSK9 measured by ELISA decreased with age in boys, but it was the opposite in girls (Baass et al. 2009).

Human plasma PCSK9 has been shown to be elevated in pregnancy at term when compared to nonpregnant, age-matched female controls, and umbilical cord blood had lower serum PCSK9 levels than maternal blood (Peticca et al. 2013). In another neonatal study, circulating PCSK9 levels showed gender-based differences and were significantly correlated with LDL-C. Their results suggest that PCSK9 could play an important role in regulating LDL-C levels during the fetal period (Araki et al. 2014).

In most populations, plasma PCSK9 levels correlate with multiple demographic and lipid and carbohydrate metabolism variables. The highest correlation is with LDL-C in nondiabetic (Alborn et al. 2007; Mayne et al. 2007; Lakoski et al. 2009) and in diabetic patients (Lambert et al. 2008). PCSK9 associates with LDL-C levels in most states of health and disease. This is consistent with the joint regulation of LDLR and PCSK9 by SREBP2 and our current understanding of PCSK9's role in LDLR degradation. Both are cleared from circulation by the same pathway. The positive correlation between serum PCSK9 and LDL-C is, however, relatively weak in contrast to the much greater effect of genetic variation in PCSK9 activity on serum LDL-C levels (Lakoski et al. 2009). Thus, serum PCSK9 levels provide a limited indication of functional PCSK9 activity. This may in future be explained in part by consideration of which of the different forms of circulating PCSK9 are measured.

The next highest correlation is with triglycerides (Lakoski et al. 2009) suggesting a possible link to the metabolism of triglyceride-rich lipoproteins as well. However, an association with TG is not consistently found, with some reports indicating no correlation (Lambert et al. 2008; Mayne et al. 2008). The correlation with serum glucose, insulin, and HOMA raises the possibility of a causative role in the metabolic syndrome.

Circulating PCSK9 in Abnormal States Associated with CVD Dyslipidemias (Familial Hypercholesterolemia, Familial Combined Hyperlipidemia)

The majority of patients with familial hypercholesterolemia (FH) have an LDLR gene mutation that results in absent or defective LDLR (FH1) or a mutation in the apolipoprotein B100 (apoB100) gene that results in a defective apoB100 (FH2). FH

Fig. 4 (continued) PCSK9 in age and sex-adjusted models ($P = 0.0004$) and after adjusting for plasma LDL-C levels ($P = 0.004$). (**c**) Relationship between nonsynonymous variants in PCSK9 and plasma levels of LDL-C in African-Americans (all, $n = 1607$) (*left*) and European-Americans (all, $n = 909$) (*right*) in the Dallas Heart Study. Individuals heterozygous for nonsynonymous variants not associated with changes in LDL-C were included in the analysis (PCSK9: G670E, A53V, V474I). Subjects taking statins were excluded.*, $P < 0.05$ (Reprinted with permission from Lakoski et al. 2009)

resulting from a GOF PCSK9 mutation (FH3) is considerably less common. Thus, serum PCSK9 levels reported in FH patients where genetic defect is not specified can be assumed to mostly reflect levels in FH1 (and to a lesser extent, FH2) and not FH3. High serum PCSK9 levels have been reported in patients with FH. Patients with homozygous FH (HoFH) have higher PCSK9 levels than patients with heterozygous FH (HeFH) who in turn have higher levels than non-FH controls. Their levels are further increased with statin therapy, more so in HeFH than in HoFH (see below) (Raal et al. 2013). As with other populations, serum PCSK9 levels correlate with serum LDL-C levels, indicating that PCSK9 plays a role in the regulation of LDL-C in FH, as in the general population. A recent report has demonstrated that elevated PCSK9 levels were equally detrimental for patients with HeFH or non-FH; a 100-ng/ml increase in PCSK9 led to an increase in LDL-C of 0.20–0.25 mmol/l in controls and HeFH alike, irrespective of their LDLR mutation (Lambert et al. 2014).

The mechanism for elevation in plasma PCSK9 in FH is likely a decrease in LDLR-mediated elimination of PCSK9. This phenomenon is supported by studies in transgenic mice overexpressing human PCSK9 showing that the clearance of serum PCSK9 is due predominantly to LDLR-mediated uptake PCSK9 (Tavori et al. 2013). However, there is also evidence for LDLR-independent pathways for clearance of PCSK9, but these remain to be more clearly demonstrated (Cameron et al. 2012; Tavori et al. 2013).

It is interesting to note that in FH, LDL-C elevation is proportionately greater than PCSK9 elevation, thereby giving rise to higher LDL-C/PCSK9 ratios. The explanation for this is not clear, but a plausible one might be that the higher PCSK9 level from diminished LDLR-related PCSK9 elimination might promote degradation of LDLR from the normal LDLR allele in HeFH, thus setting off a vicious circle of even higher circulating LDL-C (Tavori et al. 2013). Yet another explanation is that the elevation in circulating PCSK9 may not be as much as one might expect from the LDLR deficiency. Thus, the correlation between LDLR function and serum PCSK9 levels is poor, and there is overlap of PCSK9 levels among FH and non-FH patients. This may stem from the ability of dysfunctional LDLR to clear PCSK9 and from clearance of PCSK9 through non-LDLR pathways (Tavori et al. 2013).

Plasma PCSK9 levels are high in patients with familial combined hyperlipidemia (FCH), and they are correlated with plasma lathosterol levels but not markers of cholesterol absorption, suggesting that SREBP2 activation partly accounts for the high PCSK9 in FCH (Brouwers et al. 2013).

Diabetes, Glucose Homeostasis, and PCSK9

There are conflicting reports on the relationship between glucose and insulin metabolism and circulating PCSK9. In the Dallas Heart Study cohort, there was a correlation between plasma PCSK9 and plasma levels of insulin and glucose (Lakoski et al. 2009). Also, in a cohort of children and adolescents aged 9–16, there were positive associations between PCSK9 and fasting glucose, insulin, and homeostasis

model assessment of insulin resistance (HOMA-IR) (Baass et al. 2009; Dubuc et al. 2010). There is also evidence from rodent studies that *PCSK9* expression is induced by insulin and suppressed by fasting via a pathway involving SREBP1c and liver x receptor (Costet et al. 2006).

In contrast, plasma PCSK9 levels were not increased by exposure to a 24-h infusion of insulin in a hyperinsulinemic glucose clamp experiment in eight healthy subjects and eight patients with T2DM (Kappelle et al. 2011). In another study, plasma PCSK9 levels were in fact decreased by 15.4 % during an acute 3-h euglycemic-hyperinsulinemic clamp study in 82 nondiabetic postmenopausal obese patients (Awan et al. 2014). In addition, plasma PCSK9 levels were demonstrated to be not different among patients with normal glucose metabolism, impaired glucose metabolism, and T2DM (Brouwers et al. 2011). The presence of T2DM, however, was associated with steeper regression slopes for the associations with non-HDL-C and apoB. Finally, no association was found between plasma PCSK9 levels and BMI, waist circumference, fat and fat-free mass, or visceral and subcutaneous adipose tissue measured by computed tomography in abdominally obese men. There was only a trend but insignificant decrease in plasma PCSK9 levels after weight loss associated with a lifestyle modification program in abdominally obese men (Arsenault et al. 2014).

The impact of PCSK9 deficiency on glucose metabolism also remains unclear with one study showing impaired glucose tolerance and pancreatic islet abnormalities in PCSK9-deficient mice (Mbikay et al. 2010), while another failed to detect any alteration in glucose homeostasis in PCSK9-deficient mice (Langhi et al. 2009).

With these conflicting data on the association of PCSK9 with insulin and glucose metabolism, PCSK9's role in the CVD associated with various forms of dyslipidemia, especially the metabolic syndrome, remains unclear.

Plasma PCSK9 levels have been reported in only one study in patients with type 1 diabetes mellitus (T1DM), and they have lower levels than patients with T2DM. However, data from this study need verification as ~60 % of their participants were on statin therapy, which affects plasma PCSK9 levels (Cariou et al. 2010).

Chronic Kidney Disease (CKD)

In chronic kidney disease, plasma PCSK9 levels seem to be dependent on the stage of CKD. In the earlier stages of CKD (2, 3, and 4) and in nephrotic syndrome, plasma PCSK9 levels are higher in association with higher LDL-C levels (Kwakernaak et al. 2013b). However, once patients have reached CKD stage 5 and are on hemodialysis, plasma PCSK9 levels are low in association with lower LDL-C levels (Abujrad et al. 2014; Jin et al. 2014). In contrast, patients with CKD stage 5 on peritoneal dialysis have high plasma PCSK9 levels as well as high levels of LDL-C (Jin et al. 2014). Thus, plasma PCSK9 levels tend to track with LDL-C levels in CKD. The underlying mechanisms responsible for changes in plasma PCSK9 levels in CKD are unknown.

Hypothyroidism

In a group of 64 nonobese subjects, plasma PCSK9 correlated positively with serum TSH (Kwakernaak et al. 2013a). However, an acute increase of TSH levels following administration of recombinant human TSH did not raise PCSK9 levels in patients who had previously undergone total thyroidectomy and radio-ablation for thyroid cancer (Gagnon et al. 2014). This might suggest that changes in plasma PCSK9 levels are due to the effect of thyroid hormone and not TSH. In a study of 20 hyperthyroid patients, studied before and after clinical normalization, hyperthyroidism was associated with reduced circulating PCSK9, which may contribute to lower plasma LDL cholesterol in hyperthyroidism (Bonde et al. 2014).

Circulating PCSK9 and Vascular Disease

The ultimate value of PCSK9 as a biomarker is when it serves as a useful marker not just of surrogate disease markers such as serum LDL-C but of morbidity and mortality from CVD. Evidence is just starting to emerge. In a cross-sectional study of 243 patients with angiographic CVD, plasma PCSK9 levels were positively associated with the extent of coronary stenosis expressed as Gensini score, independent of age, BMI, systolic BP, smoking, family history of CVD, glucose, LDL-C, HDL-C, and TC/HDL-C and LDL-C/HDL-C ratios. There was also an association of plasma PCSK9 with the number of diseased vessels. These patients were free of lipid-lowering drug therapy for at least 3 months before entry into the study (Li et al. 2014). Another study showed that elevated serum PCSK9 levels were associated with cardiovascular (CV) events in 504 patients with stable CVD on statin therapy during a 48-month follow-up period. This study provides an added perspective in that their patients were on statin therapy, which is known to elevate serum PCSK9 concentrations. Thus, with serum LDL-C well controlled by statin therapy, serum PCSK9 remained a good predictor of CV events (Werner et al. 2014). Finally, plasma PCSK9 levels were shown to be elevated with acute myocardial infarction in two independent retrospective angiographic populations. Their patients were not on statin therapy. In one population, plasma PCSK9 levels were not associated with CVD, but in the second population, they were (Almontashiri et al. 2014). Finally, there is also evidence of an association of serum PCSK9 levels with carotid intima-media thickness (cIMT) in hypertensive subjects, independent of age, sex, total cholesterol, triglycerides, and HDL-C (Lee et al. 2013), and in non-FH patients after adjustment for lipid levels and other traditional risk factors (Huijgen et al. 2012).

In mice, gene inactivation of PCSK9 was shown to reduce atherosclerosis. There was a direct relationship between PCSK9 expression and atherosclerosis. PCSK9 overexpression is proatherogenic, whereas its absence is protective (Denis et al. 2012).

Circulating PCSK9 in Response to Lipid-Altering Drug

Statins

Statins have been shown to elevate plasma PCSK9 levels by ~30–50 % in humans (Dubuc et al. 2004, 2010; Careskey et al. 2008; Mayne et al. 2008; Cariou et al. 2010). In some studies, the rate of PCSK9 increase was shown to depend on statin dose and duration of therapy. This is not surprising as statins reduce intra-hepatocyte sterol concentrations, triggering SREBP2 synthesis which results in upregulation not only of LDLR but also PCSK9 (Horton et al. 2003; Maxwell et al. 2003). In all these studies, the association between PCSK9 levels and serum LDL-C was disrupted by statin therapy. If not for the increase in serum PCSK9 and presumably its activity in promoting LDLR degradation, the LDL-C lowering effect of statins would be greater. This line of thinking has prompted the current vigorous efforts to find strategies to inhibit the action of PCSK9.

Fibrates

The response of plasma PCSK9 to fibrate therapy is not as consistent across reports as with statin therapy. Some studies have shown an increase in patients with combined hyperlipidemia (Mayne et al. 2008; Khera et al. 2015), in patients with high triglycerides and low HDL-C (Troutt et al. 2010), and in patients with impaired glucose tolerance or T2DM (Costet et al. 2010; Noguchi et al. 2011). Other studies have shown a decrease in plasma PCSK9. This was demonstrated in a group of 115 statin-naïve type 2 diabetes mellitus (T2DM) patients in the FIELD study where fenofibrate therapy reduced PCSK9 by 8.5 % (Lambert et al. 2008). In another study of T2DM individuals already on statin therapy, add-on fenofibrate therapy reduced serum PCSK9 by 13 % along with a decrease in the VLDL particle concentration (Kourimate et al. 2008; Chan et al. 2010). While statin therapy seems to increase PCSK9 expression at a transcriptional level with increased PCSK9 mRNA, the mechanism by which fibrates alter circulating PCSK9 is not clear.

Ezetimibe

Another drug studied is the cholesterol absorption inhibitor, ezetimibe. There are reports that it further increases plasma PCSK9 levels in patients who were already on statin therapy (Davignon and Dubuc 2009; Dubuc et al. 2010), but there is another report that shows that it does not alter plasma PCSK9 levels (Berthold et al. 2013).

Niacin

The effect of niacin monotherapy on plasma PCSK9 levels has not been studied, but in studies where niacin was added to statin therapy or statin plus fibrate therapy, a decrease in plasma PCSK9 was observed. A positive association was noted between change in PCSK9 and low-density lipoprotein cholesterol levels with the addition of niacin suggesting that a portion of the LDL-C reduction seen with niacin therapy may be due to reduction in PCSK9 (Khera et al. 2015).

Bile Acid Resins

The effect of bile acid resin therapy on human plasma PCSK9 levels has not been studied, but it would be expected that it would elevate PCSK9 levels through upregulation of SREBP2. This was indirectly demonstrated in a human study in which preoperative cholestyramine treatment was associated with a 70 % increase in PCSK9 mRNA expression by liver tissue obtained at surgery (Nilsson et al. 2007).

Potential Applications to Prognosis, Other Diseases, or Conditions

Apart from PCSK9's role as a modulator of lipoprotein metabolism and CV health, there are indications that it may play an etiologic or prognostic role in other disease processes (Mbikay et al. 2013). Besides blood, PCSK9 has been demonstrated in human cerebrospinal fluid (CSF) (Chen et al. 2014). This is not surprising since PCSK9 was first recognized for its ability to enhance recruitment of undifferentiated neural progenitor cells into the neuronal lineage (Seidah et al. 2003). PCSK9 has also been shown to bind to and regulate other membrane-bound receptors such as the VLDLR (Poirier et al. 2008; Roubtsova et al. 2011) and the apolipoprotein E2 receptor (Poirier et al. 2008), both of which are highly expressed in the central nervous system (CNS). Unlike serum PCSK9 which shows a distinct diurnal pattern, CSF PCSK9 levels are constant throughout the day and are consistently lower than corresponding serum levels. The significance of PCSK9 in CSF remains to be explored.

Plasma PCSK9 has also been reported to be a late biomarker of severity in patients with severe trauma injury (Le Bras et al. 2013) and of periodontal infection (Miyazawa et al. 2012). Plasma PCSK9 levels may also serve a useful biomarker function in hepatitis C viral (HCV) infection. HCV is associated with VLDL and LDL particles in circulation, and these lipoviral particles enter hepatic cells through two receptors which are downregulated by PCSK9, namely, the LDLR and CD

81 (Labonte et al. 2009). Thus, PCSK9 may play a role in decreasing HCV uptake by the liver. However, it has not been demonstrated that plasma levels of PCSK9 serve as a useful prognostic biomarker of HCV infection.

Summary and Conclusions

PCSK9 is a secreted protein, and its role in promoting LDLR degradation occurs mostly after it has entered the circulation. Hence, it is informative to measure its concentration in blood. Circulating PCSK9 exists in its intact parent form as well as a furin-cleaved form, with loss of LDLR binding and degradation activity upon furin cleavage. Both forms may, in turn, be found free or bound to LDL particles, also with loss of LDLR binding and degradation activity upon binding to LDL particles. Thus, PCSK9 exists in several forms in circulation. Most studies reporting on serum or plasma PCSK9 levels have not distinguished among these various forms. It is theoretically important to do so, but there are currently no data to clearly indicate the need. In a different context, in individuals receiving anti-PCSK9 mAb therapy, there is yet another form of bound PCSK9, the mAb-bound form, leaving behind a lowered level of free PCSK9. Future measurements of circulating PCSK9 will need to account for the heterogeneity of PCSK9 in circulation.

GOF and LOF mutations and polymorphisms of PCSK9 are often reflected in higher and lower plasma PCSK9 levels, respectively, but this does not apply across the board.

Plasma PCSK9 has been measured in various states of health and disease, and results have enhanced our knowledge of lipoprotein metabolism in general as well as in abnormal states associated with CVD. It has also been measured in response to various forms of lipid-altering drug therapy.

It is currently not seen as a clinical marker of CVD risk or of disease progression. The few reports indicating an association between plasma PCSK9 levels and vascular disease, independent of other traditional risk factors including serum lipid levels, are the most direct evidence for a biomarker role for PCSK9. The underlying pathophysiologic mechanisms are, however, still unclear. Plasma PCSK9 may serve in future as a biomarker for the selection of patients for anti-PCSK9 therapy when drugs currently under development become available.

The alteration in plasma PCSK9 levels seen with drug therapy for dyslipidemia has also provided insight into pathophysiologic mechanisms. However, the data will need further development in order to serve as an aid to the establishment of the pharmacokinetic and pharmacodynamic properties of various forms of therapy.

In clinical practice, it is possible that it could be helpful in determining statin dosage, but its measurement is not essential as it is possible to simply titrate the dose of statin upward or downward as guided by LDL-C response.

In clinical research, plasma PCSK9 levels provide hypothesis-generating information which could help the design of basic science experiments that explore mechanisms of regulation, metabolism, and role of PCSK9.

Summary Points

- This chapter focuses on the role of proprotein convertase subtilisin-kexin 9 (PCSK9) as a key modulator of lipoprotein metabolism and cardiovascular health.
- PCSK9 is a secreted protein that promotes lysosomal degradation of low-density lipoprotein receptor (LDLR) resulting in an increase in serum low-density lipoprotein cholesterol (LDL-C).
- Circulating PCSK9 is heterogeneous as some of it undergoes furin-induced cleavage and/or binds to LDL particles, both resulting in a decrease in its LDLR degradation activity.
- Plasma PCSK9 shows a diurnal rhythm; decreases with fasting; is higher in women than men, after menopause, and in pregnancy at term; and is lower in umbilical cord blood.
- Plasma PCSK9 correlates with plasma LDL-C in most states of health and disease, indicating a role in the regulation of LDL-C levels.
- Gain-of-function and loss-of-function mutations and polymorphisms of PCSK9 are often reflected in higher and lower plasma PCSK9 levels, respectively, but this is not always the case.
- There is an association between plasma PCSK9 levels and vascular disease, independent of other traditional risk factors, but underlying pathophysiologic mechanisms are unclear.
- Statin therapy increases plasma PCSK9 levels, rendering statin therapy less effective.
- PCSK9 measurement could be helpful in determining statin dosage and in the future selection of patients for anti-PCSK9 therapy.

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Circulating Vasoactive Peptide Urotensin II and Relationships with Cardiovascular Disease

7

Isabella Albanese and Adel Schwertani

Contents

Abstract

The human vasoactive peptide urotensin II (UII) has been described as the most potent vasoconstrictor to date. Despite this, the vasoactive effects of UII have been demonstrated to be dependent on the vascular bed, the species type, and the presence of an endothelium. In recent years, several additional roles for UII in cardiovascular physiology and pathology have been established. UII has been implicated in the control of vascular tone and blood pressure as well as in cardiovascular disease states such as congestive heart failure, atherosclerosis, coronary artery disease, and pulmonary and systemic hypertension, among

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_6

others. This chapter will provide an overview of what is currently known about the physiological and pathophysiological roles of urotensin II and urotensin II-related peptide in the cardiovascular system, with particular emphasis on the implications in cardiovascular disease and the potential application of UII as a disease biomarker.

Keywords

Urotensin II • Vasoactive • Peptide • Cardiovascular • Disease • Vasoconstrictor • Hypertension • Atherosclerosis

Abbreviations

5-HT	5-hydroxytryptamine (serotonin)
ACAT-1	Acetyl-CoA acetyltransferase
ApoE	Apolipoprotein E
CHF	Congestive heart failure
CNS	Central nervous system
CSF	Cerebrospinal fluid
DAG	Diacylglycerol
EDHF	Endothelium-derived hyperpolarizing factor
EF	Ejection fraction
GDP	Guanosine diphosphate
GPCR	G-protein coupled receptor
IL-6	Interleukin 6
IP_3	Inositol 1,4,5-triphosphate
KO	Knockout
LDL	Low-density lipoprotein
LIMA	Left internal mammary artery
MEK	MAP kinase/ERK kinase 1
mRNA	Messenger ribonucleic acid
NO	Nitric oxide
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
RT-PCR	Reverse transcription polymerase chain reaction
SHR	Spontaneously hypertensive rats
TGF-β	Transforming growth factor β
UII	Urotensin II
URP	Urotensin-related peptide
WKY	Wistar Kyoto rats

Key Facts of Atherosclerosis

- Arteries are blood vessels that function to provide oxygenated blood to all parts of the body.

- Atherosclerosis is characterized as the buildup of plaque on the inner walls of arteries.
- Atherosclerotic plaques are made up of fat, cholesterol, calcium, and blood cells.
- Atherosclerosis causes the narrowing, and in severe cases, the blockage of arteries, leading to severe consequences such as heart attack and stroke.
- Studying the underlying causes of atherosclerosis is very important to prevent the incidence of heart attack, stroke, and other cardiovascular diseases.

Definitions

Atherosclerosis Hardening of the arteries caused by plaque deposition on the inner walls, leading to the narrowing or closure of the arteries.

Cardiac Remodeling Changes to the shape and structure of cardiovascular tissue in response to injury.

Cardiomyocyte Cardiac muscle cell found within the walls of the heart.

Congestive Heart Failure Weakening of the heart muscle that results in fluid accumulation in the lungs and surrounding tissue.

Ejection Fraction The percentage of blood volume that is ejected from the blood with each contraction.

Endothelium Single layer of cells surrounding various organs and openings (ex: blood vessels).

Hyperlipidemia Abnormally high lipid content in the blood.

Hypertension Abnormally high blood pressure.

Urotensin II Neuropeptide with potent vasoconstrictive effects that has been implicated in several cardiovascular pathologies.

Vasoconstriction The narrowing of blood vessels.

Introduction

The vasoactive urotensin II peptide was first biologically characterized by Bern et al. in 1967 (Bern et al. 1985) and was initially isolated in the teleost fish *Gillichthys mirabilis* (Pearson et al. 1980). Although originally believed to be present only in fish (Prosser et al. 2006), the human form of urotensin II and homologues from other animals have been isolated (Coulouarn et al. 1998;

Elshourbagy et al. 2002). Human UII is an 11-amino acid peptide that is derived from cleavage of two possible larger precursor prepropeptides, one of 124 amino acids in length and the other of 139 amino acids (Douglas and Ohlstein 2000). Of functional significance is the six-amino acid cyclic structure that has been evolutionarily conserved from fish to mammals (Bern et al. 1985; Elshourbagy et al. 2002) and is believed to be responsible for the biological activity of UII (Barrette and Schwertani 2012). This cyclic structure is essential for UT receptor binding and activation (Flohr et al. 2002). Although its receptor was unknown at the time of UII peptide isolation, using a reverse pharmacological technique, Ames et al. characterized an orphan G-protein coupled receptor (GPCR), GPR14, as the endogenous UII receptor, and this receptor was renamed the UT receptor by the International Union of Pharmacology (Ames et al. 1999). Another ligand for the UT receptor named Urotensin II-related peptide (URP) was discovered in rat brain and has been isolated in humans and mice (Sugo et al. 2003). URP is an eight-amino acid peptide with a cyclic carboxy terminus identical to that of UII (Sugo et al. 2003). Although previously thought to be endothelin-1, urotensin II has been demonstrated to be the most potent physiological vasoconstrictor peptide (Ames et al. 1999).

Potential Applications to Prognosis, Other Diseases, or Conditions

After UII was isolated from the urophysis of the teleost fish *Gillichthys mirabilis,* it was subsequently found that in the CNS of tetrapods, UII expression is highest in brainstem and spinal cord motor neurons (Vaudry et al. 2010). In human tissues, UT receptor density is highest in the cerebral cortex and skeletal muscle (Coulouarn et al. 1998; Maguire et al. 2000). The UT receptor is widely expressed in the rat brain (cortex, lateral septal nucleus, thalamic nuclei, abducens nucleus, cerebellum) and spinal cord (white and grey matter and dorsal root ganglion), with the highest density of receptors in the abducens and lateral septal nuclei of the brain (Maguire et al. 2008). This robust expression across the CNS implies a potential neuromodulatory role for the UT receptor system. Studies in the rat brainstem have identified the presence of UII in cholinergic motor neurons (Dun et al. 2001). Studies in the human brainstem and spinal cord have also found UII immunoreactivity in ventral horn motor neurons (Chartrel et al. 2004). A recent study demonstrated both UII and URP mRNAs to be expressed in mouse brainstem and spinal motor neurons (Dubessy et al. 2008). In this study, UII and URP were similarly expressed in the mouse skeletal muscle, testes, vagina, and gall bladder, while only URP mRNA was detected in the seminal vesicles, heart, colon, and thymus. UT receptor mRNA was widely expressed in various mouse tissues with highest expression in skeletal muscle and prostate (Dubessy et al. 2008). Human UII is also robustly expressed in the cardiovascular system (Maguire et al. 2000) and the UT receptor is predominantly expressed in the heart and smooth muscle (Douglas and Ohlstein 2000). Prepro-UII mRNA expression has been found in human vascular smooth muscle cells (Douglas et al. 2002), endothelial cells (McDonald et al. 2007),

and rat cardiac fibroblasts (Tzanidis et al. 2003), suggesting that mature UII formation occurs within the cardiovascular system (Russell 2004). UII and UT display strong expression in the peripheral vasculature and heart (Matsushita et al. 2001) and more specifically, UT is expressed in the atria and ventricles, in endothelial cells across the vasculature, and in arterial but not venous smooth muscle cells (Ames et al. 1999; Liu et al. 1999). While low-plasma UII concentrations are consistently observed in healthy patients, UII in plasma is significantly elevated in cases of cardiac dysfunction, such as congestive heart failure (CHF) (Russell et al. 2003). Specifically, plasma UII in CHF patients is elevated in the aortic root compared to the pulmonary artery (Russell et al. 2003). Charles et al. provided further evidence of UII release from the heart in a study measuring the arteriovenous gradient of UII concentration across the heart in an anesthetized sheep model (Charles et al. 2005). UII and URP mRNA expression is significantly elevated in the atrium of spontaneously hypertensive rates (SHR) compared to age-matched WKY rats, while UT mRNA expression is elevated in the ventricles of SHR (Hirose et al. 2009). In a study of UT receptor localization in human tissues, there was little binding of radiolabeled UII in the atria, cardiac conduction system, and lungs. There was a low UT receptor density in human coronary artery smooth muscle compared to rat aorta and no significant difference between calcified and normal blood vessels (Maguire et al. 2000). Subsequent confocal laser microscopy studies show the presence of the UT receptor in human vascular smooth muscle cells and cardiac myocytes of coronary arteries, saphenous veins, and left internal mammary arteries (LIMA). Lower levels of the UT receptor were present in renal, adrenal, and pulmonary vessels as well as in vascular endothelial cells of human coronary arteries, saphenous veins, and LIMA (Maguire et al. 2008). UII, URP, and UT levels have been found to be upregulated in various cardiovascular disease states, including but not limited to systemic and pulmonary hypertension, atherosclerosis, and congestive heart failure.

The renal system is another important site of UII production. Several studies have demonstrated high human prepro-UII mRNA expression in the kidneys (Coulouarn et al. 1998). In normal human kidneys, UII is present in the epithelial cells of tubes and ducts, with greatest intensity in distal convoluted tubules (Shenouda et al. 2002). Elevated urine UII concentration in normal individuals whose plasma UII levels were undetectable suggests the renal production of UII (Matsushita et al. 2001). In addition to the studies demonstrating UII immunoreactive staining in tubular epithelial cells, immunoreactivity has also been detected in renal capillary endothelial cells (Shenouda et al. 2002). In a study assessing the binding density of radiolabeled UII in various human tissues, there was low-density binding in the kidney cortex suggesting the presence of low levels of the UT receptor in this region (Maguire et al. 2000). A recent study of spontaneously hypertensive rats (SHR) found that while UII and UT receptor mRNA expression did not differ in the kidneys of pre-hypertensive SHR compared to normotensive WKY rats, URP mRNA expression was fourfold higher, suggesting a potential role for URP in spontaneous hypertension (Forty and Ashton 2013). In older SHR, both URP and UT mRNA expression are elevated compared to age-matched WKY rats (Hirose et al. 2009).

The liver is also a documented site of UII production. There is elevated plasma UII concentration across the liver in cirrhotic patients, particularly in the hepatic vein compared to the hepatic portal vein (Heller et al. 2002). A later study in patients with chronic liver disease revealed their elevated serum UII to be associated with the severity of the liver disease and the extent of portal hypertension (Kemp et al. 2007). In addition to plasma UII being higher in cirrhotic liver patients, it was recently found that UT expression at the mRNA and protein level is significantly elevated in the livers of patients with cirrhosis and portal hypertension compared to normal livers (Liu et al. 2010). Taken together, these findings suggest that the liver is a source of UII production, particularly in pathophysiological conditions where the UT receptor system appears to be playing a role in cirrhosis and portal hypertension.

The most studied aspect of urotensin II is its vascular activity (Barrette and Schwertani 2012). Both endothelium-dependent vasorelaxation and endothelium-independent vasoconstriction mediated by UII have been demonstrated in rat-isolated aorta (Gibson 1987). Despite being characterized as the most potent isolated vasoconstrictor, the constrictive effects of UII are variable and appear to be dependent on the vascular bed (Itoh et al. 1987) and the species from which it is isolated (Douglas et al. 2000). URP has been demonstrated to be a less potent vasoconstrictor (Chatenet et al. 2004) and vasodilator (Prosser et al. 2006) than UII in rats, despite having the same binding affinity to the UT receptor.

In addition to its potent vasoactive effects, urotensin II has several other crucial roles in cardiovascular physiology and pathophysiology, many of which have only begun to be identified in recent years. Under normal physiological conditions, UII-UT binding is integral in the control of vascular tone, blood pressure, and maintaining blood glucose levels (Douglas and Ohlstein 2000; Douglas et al. 2000). UII's physiological roles also include mediating the release of endothelial-derived vasodilators, such as nitric oxide, and thus controlling the contraction and relaxation of vascular smooth muscle cells (Gibson 1987). The pathological roles for the UT receptor system are still emerging. There is evidence implicating this system in conditions such as congestive heart failure, atherosclerosis and coronary artery disease, and both systemic and pulmonary hypertension, cirrhosis, and chronic renal failure, among others. There is also evidence suggesting that positive and negative inotropy, arrhythmias, cardiomyocyte hypertrophy, vascular smooth muscle cell proliferation, extracellular matrix production, and hyperpermeability of endothelial cells are among some of the other cardiovascular effects of the urotensin system in pathophysiological conditions (Russell 2004).

Regulation of Vascular Tone

Initial studies of UII demonstrated a potent vasoconstrictor activity on isolated rat arteries (Gibson et al. 1986). Similarly, UII causes vasoconstriction on human coronary, mammary, and radial arteries with their epithelia removed (Maguire et al. 2000). In an in vivo study in human subjects, Böhm and Pernow observed potent vasoconstrictor activity upon local administration of UII (Bohm and Pernow

2002). However, a great amount of heterogeneity has been observed among vasoactive responses to UII among vascular beds from different species as well as different regions within an animal. Potent vasodilation in response to UII has been frequently observed (Gibson 1987; Desai et al. 2008). Interestingly, in a review by Desai et al., it is suggested that UII's vasoactivity is dependent on blood vessel diameter (Desai et al. 2008). Smaller arteries of 0.07 mm in diameter, whose responses are thought to be more endothelium-mediated, vasodilate in response to UII, while arteries 0.07–0.25 mm in diameter show a more attenuated response and large vessels with a 0.25 mm diameter, whose responses are thought to be more smooth muscle-mediated, show no response (MacLean et al. 2000; Stirrat et al. 2001). Properties of the signal transduction cascade of the UT receptor system could be contributing to the differential vasoactive effects observed of UII. When the UII peptide binds the UT receptor in vascular smooth muscle cells, this leads to dissociation of the αβγ G-protein complex and activation of $G\alpha_{q/11}$. The activated $G\alpha_{q/11}$ causes the phospholipase C-mediated hydrolysis of PIP_2 (phosphatidylinositol 4,5-bisphosphate) into IP_3 (inositol 1,4,5-triphosphate) and DAG (diacylglycerol). IP_3 can then bind to its receptors on endoplasmic/sarcoplasmic reticula, causing the increase in intracellular Ca^{2+} underlying contraction. In the endothelium-mediated vasodilation caused by UII, UT receptor activation on the endothelium leads to the release of nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF), causing vasodilation (McDonald et al. 2007). Differences in expression of the UT receptor in vessels of different sizes and locations may also contribute to the variability in response to UII (Onan et al. 2004). Interestingly, through its two receptor subtypes, endothelin-1 also mediates endothelium-independent vasoconstriction and endothelium-dependent vasodilation (Maguire et al. 2000). Despite being the most potent vasoconstrictor, urotensin II is often described as having the most variable responses (Russell and Molenaar 2004) and the least efficacy compared to other vasoconstrictors such as endothelin-1, angiotensin II, and noradrenaline (Maguire et al. 2000).

Regulation of Myocardial Contractility

The direct effects of UII on human myocardial contractility were studied in strips of myocardium isolated from human patients that were stimulated to contract at 60 beats/min. When UII was applied, it increased contractile force in the right atrium and right ventricle and was identified as the most potent inotropic agent to date, surpassing endothelin-1, serotonin, and noradrenaline (Russell et al. 2001). UII displayed similar contractile activity on myocardial strips isolated from rats (Gong et al. 2004). In vivo studies in cynomolgus monkeys and anesthetized rats have shown that acute systemic infusion of UII mediates a drop in mean arterial blood pressure, which is contradictory to UII's supposed positive inotropic effects (Ames et al. 1999; Hassan et al. 2005). A potential explanation for the observed variability in contractile responses to UII administration is that the drop in blood pressure and

contractility could be due to coronary artery constriction caused by UII (Desai et al. 2008).

Role in Hypertension

UII's status as the most potent endogenous vasoconstrictor suggests a potential role in essential hypertension (Desai et al. 2008). Hypertensive patients were found to have elevated levels of urotensin II compared to normotensive controls in a study by Cheung et al. where UII was directly related to systolic blood pressure (Cheung et al. 2004). Hypertensive survivors of myocardial infarction also had significantly elevated plasma UII levels after exercise compared to similar patients without hypertension (Rdzanek et al. 2006). A study by Thompson et al. was first to measure cerebrospinal fluid (CSF) UII levels and found CSF UII levels to be lower than those of plasma and remarkably, found a significant positive correlation between CSF UII concentrations and mean arterial blood pressure (Thompson et al. 2003). After demonstrating that URP and UT expression are upregulated in the kidneys of rats with chronic renal failure or hypertension (Mori et al. 2009), Hirose et al. went on to examine the gene expression of UII, URP, and UT in the heart and aorta of hypertensive rats and found an increased expression of the entire UT system (Hirose et al. 2009). A development in the study of the UT receptor system in hypertension is the study by Behm et al. which reports a hypertensive cat model to be useful in monitoring classical systemic hypertensive responses and the effects of urotensin II administration and UT receptor antagonism on these parameters (Behm et al. 2004). The localization of urotensin II in the brain stem in the rat and in the human central nervous system also point to potential involvement in the central regulation of blood pressure (Ames et al. 1999; Dun et al. 2001). Supporting UII's possible role in blood pressure regulation, microinjection of urotensin II into the brainstem area A1 of anesthetized rats led to dose-dependent decreases in heart rate and blood pressure, while microinjection into the paraventricular and arcuate nuclei led to increases in blood pressure and heart rate (Lu et al. 2002). Additionally, Lin et al. found that central urotensin II increases blood pressure of normotensive rats via sympathetic activation (Lin et al. 2003). They subsequently found central urotensin II-mediated increases in blood pressure to be even greater in hypertensive rats (Lin et al. 2003). In addition to the diverse effects on the peripheral vasculature, these studies point to UII having an important role in the CNS.

Given the accumulating evidence for the UT receptor system's role in systemic hypertension, studies have emerged on the roles in pulmonary hypertension. Human UII was found to be a potent vasoconstrictor in pulmonary arteries isolated from hypoxic rats, and this response increased at the onset of pulmonary hypertension (MacLean et al. 2000). On the other hand, pulmonary arteries isolated from humans did not respond to UII (MacLean et al. 2000; Stirrat et al. 2001). While plasma and lung concentrations of UII are unchanged in hypoxic rats, there was an observed pulmonary pressure-induced increase in UT receptor expression in the right ventricle (Zhang et al. 2002). In another rat model of pulmonary hypertension, UII

immunoreactive staining was upregulated in endothelial cells and smooth muscle cells of small pulmonary arteries (Qi et al. 2004). In order to determine a more direct role for the UT receptor system in pulmonary hypertension, a recent study by Onat et al. used UII antagonist palosuran in a rat model for pulmonary hypertension and observed significant decreases in mean pulmonary arterial pressure, right ventricular hypertrophy, and right ventricular myocardial infarction (Onat et al. 2013). There are limitations to this study as the palosuran inhibitor also decreased endothelin-1 and TGF-β. More selective inhibitors and further research will continue to provide insight into the direct roles of the UT receptor system in pulmonary hypertension and other cardiovascular pathologies.

Role in Atherosclerosis

Atherosclerosis is a leading cause of death in Western societies and is a major contributing factor to several cardiovascular diseases; thus, determining the role of the UT receptor system in atherosclerosis is a critical area of study. Bousette et al. were first to demonstrate that atherosclerotic lesions of the human carotid arteries and aorta have increased expression of UII and UT compared to healthy vessels (Bousette et al. 2004). More specifically, using immunohistochemistry, strong UII immunoreactivity was observed in endothelial, smooth muscle, and inflammatory cells, particularly in the intima, in both carotid and aortic plaques. Using quantitative real-time RT-PCR analysis, they demonstrated that UII production mainly occurs in leukocytes, while UT expression is mediated primarily by monocytes and macrophages (Bousette et al. 2004). This suggests that inflammatory cells play an important role in the UT receptor system's atherosclerotic function. In a later study, the same group found elevated levels of UII mRNA and protein in atherosclerotic coronary arteries compared to normal coronary arteries, with UII expression highest in endothelial cells in areas in inflammatory or fibrofatty lesions (Hassan et al. 2005). Similarly, in another study, UII expression was reported to be localized to areas of macrophage infiltration in atherosclerotic coronary arteries (Maguire et al. 2004). In several studies, elevated UII levels have also been observed in human plasma of patients with atherosclerosis. UII alone and synergistically with oxidized low-density lipoprotein (LDL) also enhances vascular smooth muscle cell proliferation, a key process for the intimal thickening stage of atherosclerosis (Watanabe et al. 2001). This is of great clinical significance as oxidized LDL is a major contributing factor to atherosclerotic plaque formation. Furthermore, UII has also been linked to increased foam cell formation in atherosclerosis (Watanabe et al. 2005). Acyl-coenzyme A: cholesterol acyltransferase-1 (ACAT-1) is a key enzyme in cholesterol homeostasis and functions to convert intracellular free cholesterol into cholesterol ester for storage in lipid droplets. ACAT-1 is important in the formation of foam cells, which form early in atherosclerotic lesions by macrophages continuously taking up oxidized LDL via scavenger receptors. The accumulation of these macrophage-derived foam cells contributes to the necrotic fibrofatty cores seen in atherosclerotic lesions (Bousette and Giaid 2006). UII's stimulating effects on foam cell formation seem to involve

various intracellular signaling pathways as they were inhibited by selective UT antagonist urantide, PKC inhibitor rottlerin, MEK inhibitor PD98059, Rho kinase inhibitor Y27632, c-Src protein tyrosine kinase inhibitor PP2, and G-protein inactivator GDP-beta-S (Watanabe et al. 2005). In recent years, significant evidence has begun to emerge about the roles of the UT receptor system in atherosclerosis by the use of genetic inhibition and the development of new pharmacological inhibitors. A recent study by You et al. demonstrate that UII gene deletion in atherosclerotic mice as well as the use of UT receptor antagonist SB657510A ameliorate many features of atherosclerosis including reducing serum cytokines and adipokines, improving aortic atherosclerosis, reducing weight gain and fat deposition, decreasing blood pressure, and improving glucose tolerance (You et al. 2012). After observing increased UII expression in diabetes-associated atherosclerotic mice and humans, Watson et al. demonstrated that the same UT receptor antagonist SB657510 attenuated diabetes-associated plaque development (Watson et al. 2013). Interestingly, Bousette et al. observed that genetic deletion of the UT receptor on ApoE knockout mice (a model for atherosclerosis) increased atherosclerosis as well as serum insulin and lipids in these mice. It is suggested that UT receptor deletion in these mice downregulates ACAT-1 expression, ultimately decreasing receptor-mediated lipoprotein uptake in the liver. This increases hyperlipidemia, decreases hepatic steatosis, and along with UT-KO-associated hypertension, is thought to contribute to the increase in atherosclerosis seen in these mice (Bousette et al. 2009). The further development of pharmacological agents capable of interfering with the UT receptor system and their use in animal models will contribute to a better understanding of the roles of urotensin II and urotensin II-related peptide in atherosclerosis as well as the development of novel therapeutic approaches (Figs. 1, 2, and 3).

Role in Heart Failure

Under physiological conditions, UII expression is strongest in the central nervous system but is significantly increased in the heart in cardiovascular disease states (Bousette and Giaid 2006). Both UII and UT expression increase significantly in patients with end-stage congestive heart failure (CHF), particularly in cardiomyocytes and to a lesser extent in vascular smooth muscle cells, endothelial cells, and inflammatory cells (Douglas et al. 2002). This study was the first to demonstrate a potential correlation between UII levels and cardiac dysfunction as they observed an inverse relationship between UII levels and ejection fraction (EF) (Douglas et al. 2002). This increase in UII seen in cardiac dysfunction is supported by several studies demonstrating elevated plasma UII levels in CHF patients (Russell et al. 2003). In studies where disease data was separated by etiology, UII levels in plasma increased similarly in both ischemic and non-ischemic CHF (Douglas et al. 2002; Russell et al. 2003). The inverse relationship between UII and EF in CHF patients is supported by additional studies by Gruson et al. (2006). Conversely, there have also been studies showing no difference in plasma UII in CHF patients compared to normal controls (Dschietzig et al. 2002), potentially due to differences in patient populations (Bousette and Giaid 2006).

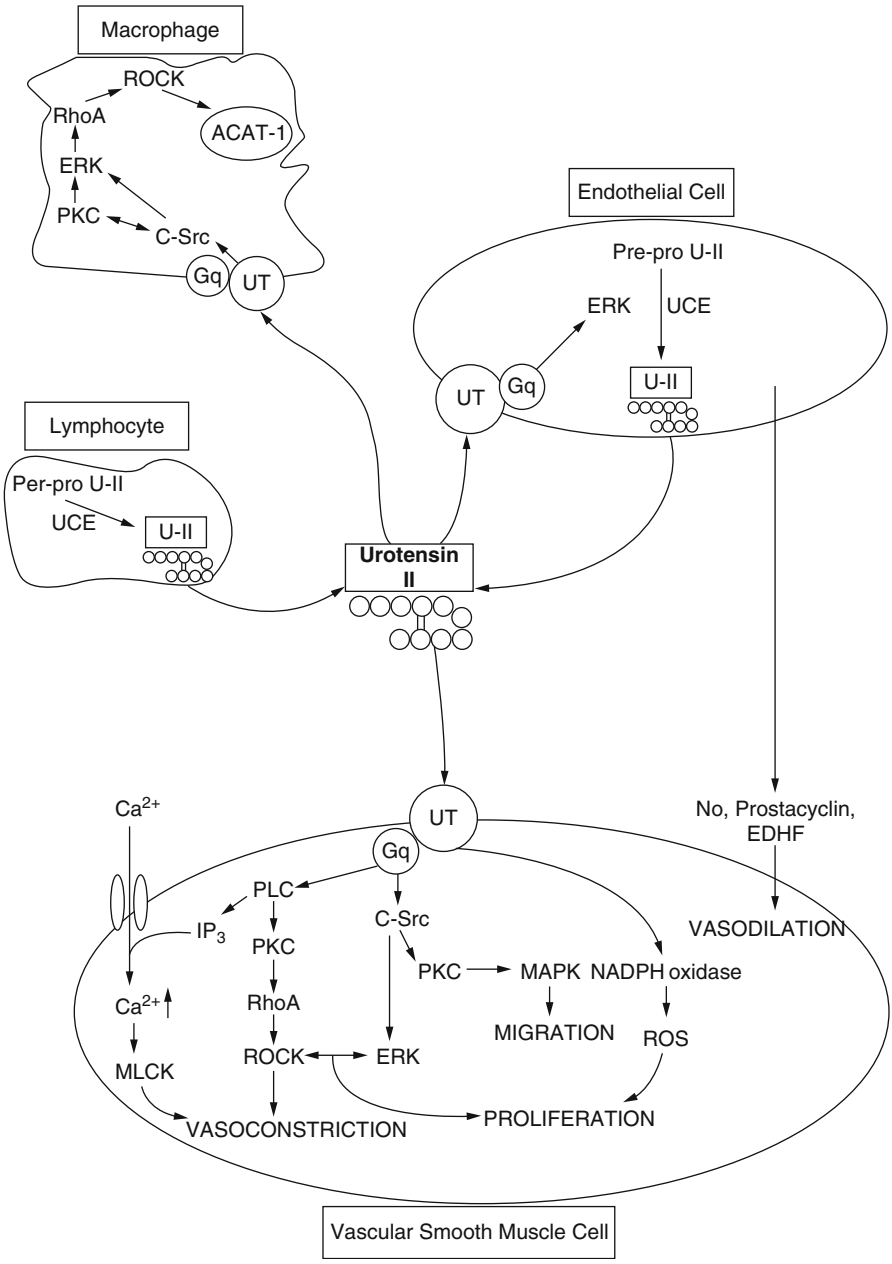

Fig. 1 Biochemical signaling pathways mediated by UII (With permission from Ross et al. (2010). Copyright © 2010, The American Physiological Society)

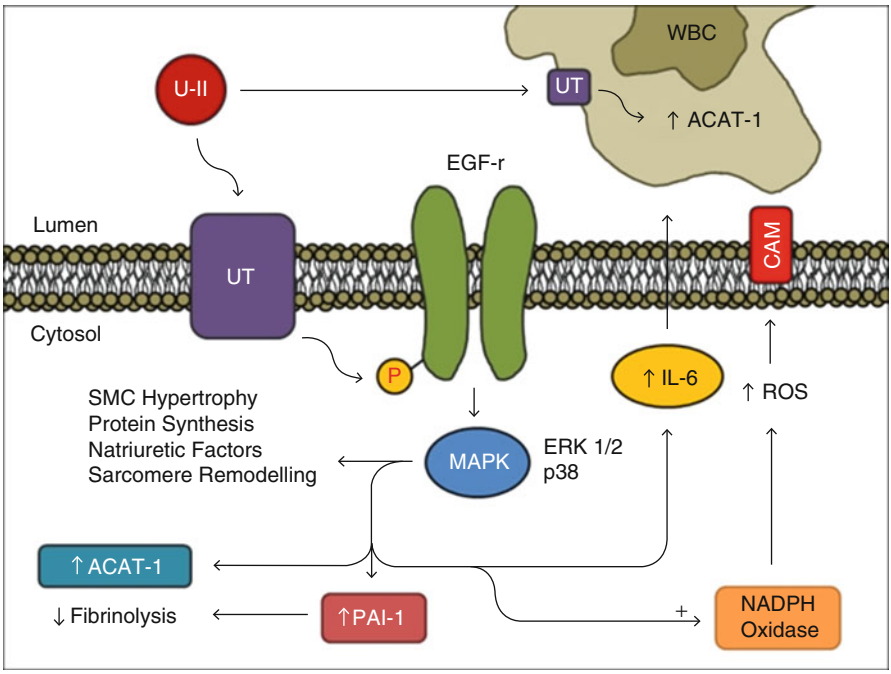

Fig. 2 Roles for UII in atherosclerosis (From Tsoukas et al. (2011))

Recently, Jani et al. have developed a solid phase extraction technique such that both plasma UII and URP can be differentially isolated and assayed separately (Jani et al. 2013). Given the structural similarity between UII and URP, this ensures the specificity of both measurements. Using this newly developed technique, Jani et al. observed significant increases in both UII and URP plasma levels in patients with acute heart failure compared to healthy controls, suggesting roles for the entire UT receptor system in acute heart failure (Jani et al. 2013). This is supported by a study by Nakayama et al. which demonstrates an increase in gene expression of URP, UII, and UT in the hearts of rats with CHF (Nakayama et al. 2008). While there is accumulating evidence associating plasma urotensin II levels with cardiac dysfunction, current studies aim to elucidate functional roles for the UT receptor system in heart failure. In a study by Lim et al., a noninvasive iontophoresis technique was used to examine the effects of UII on cutaneous blood flow in normal and CHF patients. While UII administration caused vasodilation in normal patients, in patients with CHF, UII caused vasoconstriction, indicated by reduced blood flow in skin microcirculation (Lim et al. 2004). Since endothelial dysfunction is a common feature of CHF (Katz 1997), several studies have shown UII to have differential endothelium-dependent and independent vasoactive effects (Gibson 1987). This suggests that the functional state of the endothelium in CHF is a critical consideration in determining the effect of the UT receptor system in CHF (Table 1). Using selective UT receptor antagonist SB-611812 in rats after coronary artery ligation,

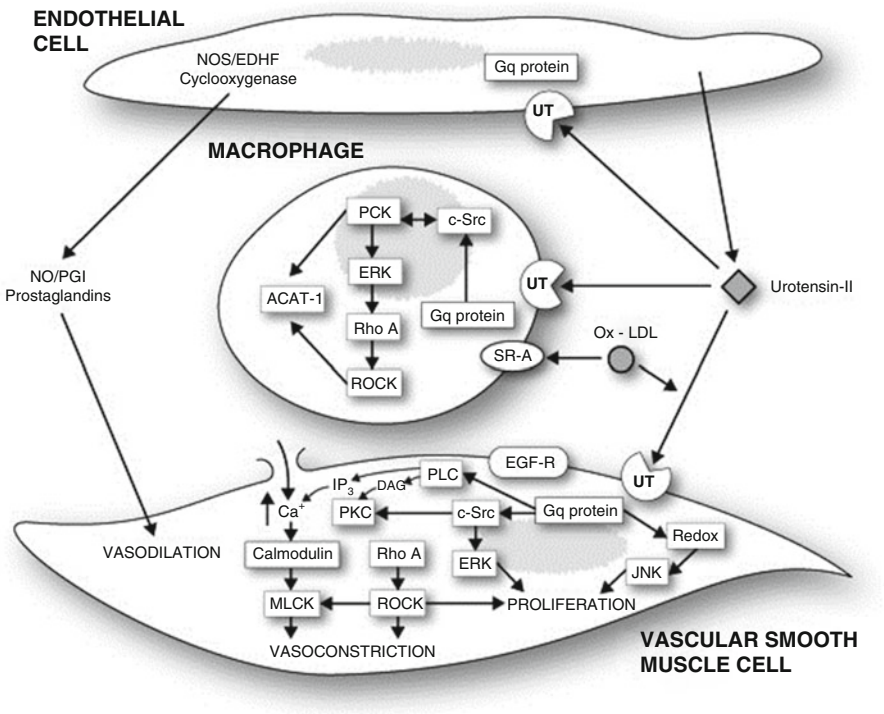

Fig. 3 Mechanisms of UII action in endothelial cells, macrophages, and VSMCs (From Papadopoulos et al. (2008))

Bousette et al. demonstrated significantly improved cardiac dysfunction (Bousette et al. 2006). Specifically, blocking the UT receptor led to decreases in left ventricular end diastolic pressure, lung edema, right ventricular systolic pressure, central venous pressure, cardiomyocyte hypertrophy, and ventricular dilatation (Bousette et al. 2006). A subsequent study in a rat model of ischemic CHF showed that SB-611812 administration attenuates cardiac remodeling (Bousette et al. 2006). SB-611812-mediated UT receptor blockage decreased myocardial fibrillar collagen deposition and led to a reduced ratio of collagen type I to type III (Bousette et al. 2006), which has been previously linked to decreased diastolic dysfunction (Nishikawa et al. 2001). This is consistent with previous work demonstrating the fibrotic effects of UII in vitro and in vivo. Tzanidis et al. demonstrated that incubation of cardiac fibroblasts with UII led to increased expression of fibronectin, type I, and type III procollagen mRNA and significant collagen peptide synthesis upon overexpression of recombinant UT receptor (Tzanidis et al. 2003). This suggests that UII's fibrotic role in myocardial remodeling would be enhanced in diseased states where increased UT has been repeatedly demonstrated (Russell 2004). There is an increasing amount of evidence implicating UII in cardiac

Table 1 Summary of UII plasma levels in disease and control

Technique	Disease studied	Sample details	Group makeup		Plasma UII levels, pmol/l		Reference no.
			Control	Experimental	Control	Experimental	
RIA	Healthy state	VA	15 m, 0 f; 37 years	0	12 ± 3		142
RIA	Blood pressure	VA	10 m, 0 f; 42 years	0	16 ± 1		2
RIA	HT	VA	28 m, 34 f; 54 years	32 m, 30 f; 57 years	8.8 ± 0.9	13.6 ± 1.4	24
RIA	CHF	AR	15 m, 3 f; 59 years	18 m, 3 f; 57 years	16.3 ± 4.4	166.2 ± 49.5	102
RIA	CHF		$n = 88$; 21–76 years	$n = 74$; 52–89 years	1.9 ± 0.9	3.9 ± 1.4	101
RIA	CHF		$n = 20$	$n = 45$	3.1 ± 0.9	1.0 ± 0.8	150
RIA	Angina	VA	40 age, sex matched	69 m, 25 f; 64 years	1.35 ± 0.3	1.5 ± 0.46	22
RIA	ACS	VC	$n = 21$; 46 years	$n = 59$; 62 years	2,373 ± 2,804	1,819 ± 1,164	59
RIA	CAD	CA	14 m, 10 f; 63 years	45 m, 8 f; 67 years	3.5 ± 0.8	1.3 ± 0.5	146
RIA	CAD	VC	$n = 20$	$n = 58$	2.6 ± 0.9	1.2 ± 0.7	37
RIA	DM w/o proteinuria	VA	12 m, 10 f; 15–63 years	7 m, 9 f; 20–78 years	4.4 ± 2.0	7.8 ± 0.6	127
RIA	DM w/ proteinuria	VA	12 m, 10 f; 15–63 years	3 m, 3 f, 46–77 years	4.4 ± 2.0	7.3 ± 0.9	127
RIA	Renal failure	FNS	13 m, 11 f; 19–58 years	8 m, 4 f, 35–78 years	4.4 ± 1.0	13.1 ± 3.1	129
RIA	MCNS		$n = 26$; 2–7 years	$n = 16$; 2–7 years	39.4 ± 27.7	14.5 ± 10.4	6
RIA	DN	VA	8 m, 2 f; 18–69 years	2 m, 4 f; 57 years	5.2 ± 0.4	15.9 ± 2.2	128
RIA	Cigarette use	VC	$n = 20$; 37 years	$n = 20$; 36 years	1.2 (0.7–1.6)	1.9 (1.3–2.5)	42

ELISA	CHF (systolic)		142 m, 78 f; 61 years	89 m, 37 f; 63 years	6.6 (3.1–42.6)	22 (31.–49.2)	89
ELISA	IC	VC	7 m, 2 f; 63 years	9 m, 4 f, 70 years	1,357 ± 463	3,474 ± 521	70
ELISA	CA		31 m, 0 f; 61 years	50 m, 0 f; 62 years	1,653 ± 862	5,681 ± 5,465	118
ELISA	Acute MI		$n = 21$; 60 years	$n = 129$; 64 years	0.42 (0.29–4.27)	1.4 (0.29–34.5)	65
ELISA	HT after MI, rest	VA	17 m, 6 f; 56 years	13 m, 4 f; 58 years	39,500 ± 27,405	45,534 ± 25,967	100
ELISA	HT after MI, exercise	VA	17 m, 6 f; 56 years	13 m, 4 f; 58 years	53,192 ± 34,891	70,494 ± 29,102	100
ELISA	Portal HT	PV	8 m, 7 f; 31–63 years	29 m, 21 f; 41–76 years	2,592 (72–8,640)	8,856 (1,152–29,808)	45
ELISA	Migraine	VC	10 m, 17 f; 19 years	11 m, 16 f; 15 years	0.5 (25 % = 0.3, 75 % = 0.6)	0.3 (25 % = 0.06, 75 % = 0.6)	10
EIA	CHF	FNS	9 m, 4 f; 55 years	7 m, 4 f; 55 years	60.4 ± 17.3–79.8 ± 28.8	79.1 ± 28.8–93.5 ± 23.0	34
EIA	CHF (rest)	VC	5 m, 5 f; 54 years	25 m, 7 f; 60 years	2,365.9 ± 365.3	2,150.2 ± 793.9	66
EIA	CHF (peak exercise)	VC	5 m, 5 f; 54 years	25 m, 7 f; 60 years	2,310.5 ± 854.3	2,202.6 ± 852.2	66
EIA	CAD	PA	32 m, 4 f; 60 years	31 m, 5 f; 67 years	729.9 ± 467.4	1,086.6 ± 637.1	46
EIA	Hemodialysis	VA, HM	131 m, 36 f; 58 years	106 m, 85 f	2,373 (1,725–3,307)	4,674 (2,085–8,269)	155
ILMA	Cigarette use	VC	$n = 20$; 37 years	$n = 20$; 36 years	0.3 (0.3–0.7)	0.3 (0.3–0.5)	42

Values are means ± SE; *n* number in group; numbers in parentheses are range, *m* male, *f* female, *year* mean age, *w* with, *w/o* without, *RIA* radioimmunoassay, *MI* myocardial infarction, *VA* venous arm, *CHF* congestive heart failure, *VC* venous catheter, *EIA* enzyme immunoassay, *HT* hypertension, *PA* pulmonary artery, *ILMA* immunoluminometric assay, *CAD* coronary artery disease, *CA* coronary angiography, *DM* diabetes mellitus, *FNS* from numerous sites, *MCNS* minimal change nephrotic syndrome, *HM* hemodialysis, *ACS* acute coronary syndrome, *PV* portal venous, *IC* ischemic cardiomyopathy, *AR* aortic root, *CA* carotid atherosclerosis, *DN* diabetic nephropathy

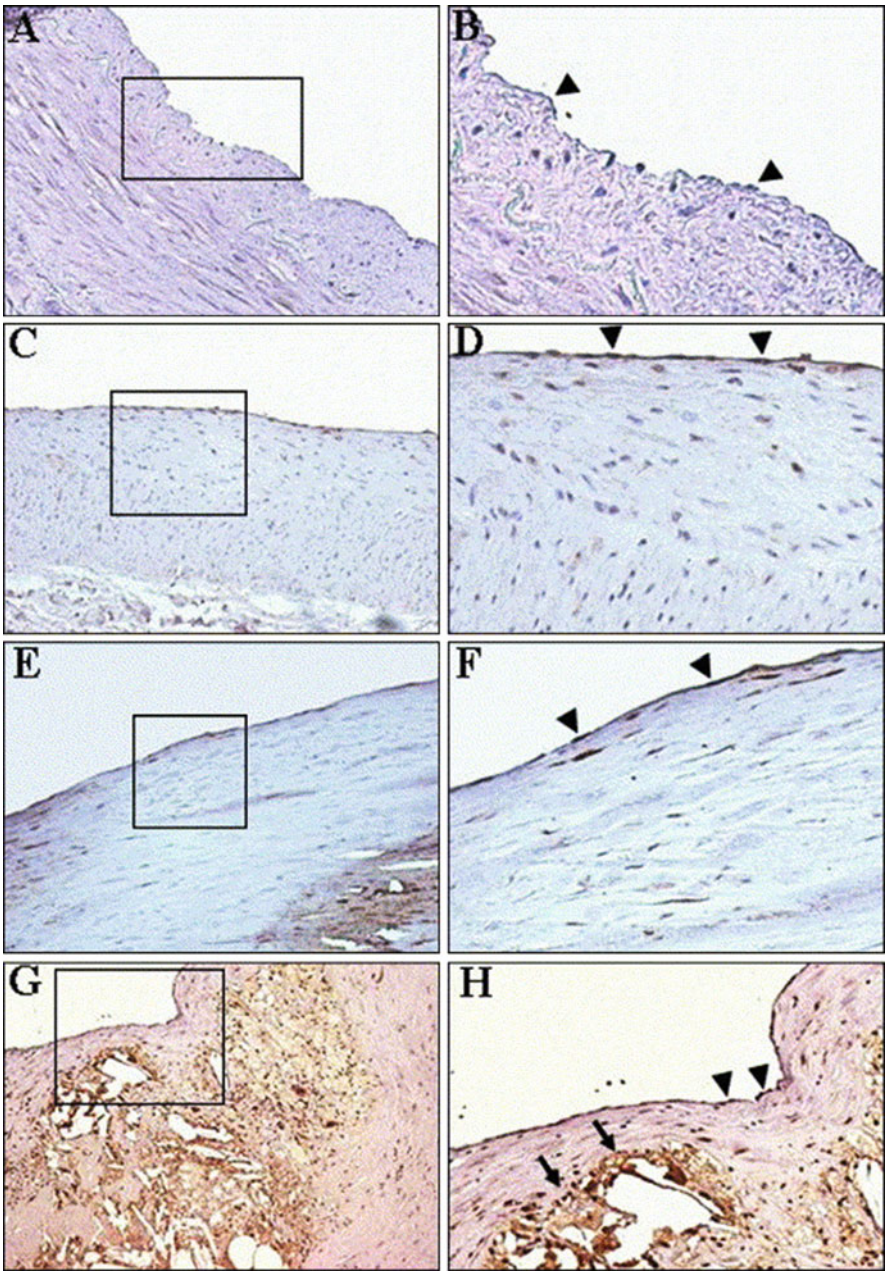

Fig. 4 Immunohistochemical localization of UII in normal and atherosclerotic coronary arteries (From Hassan et al. (2005))

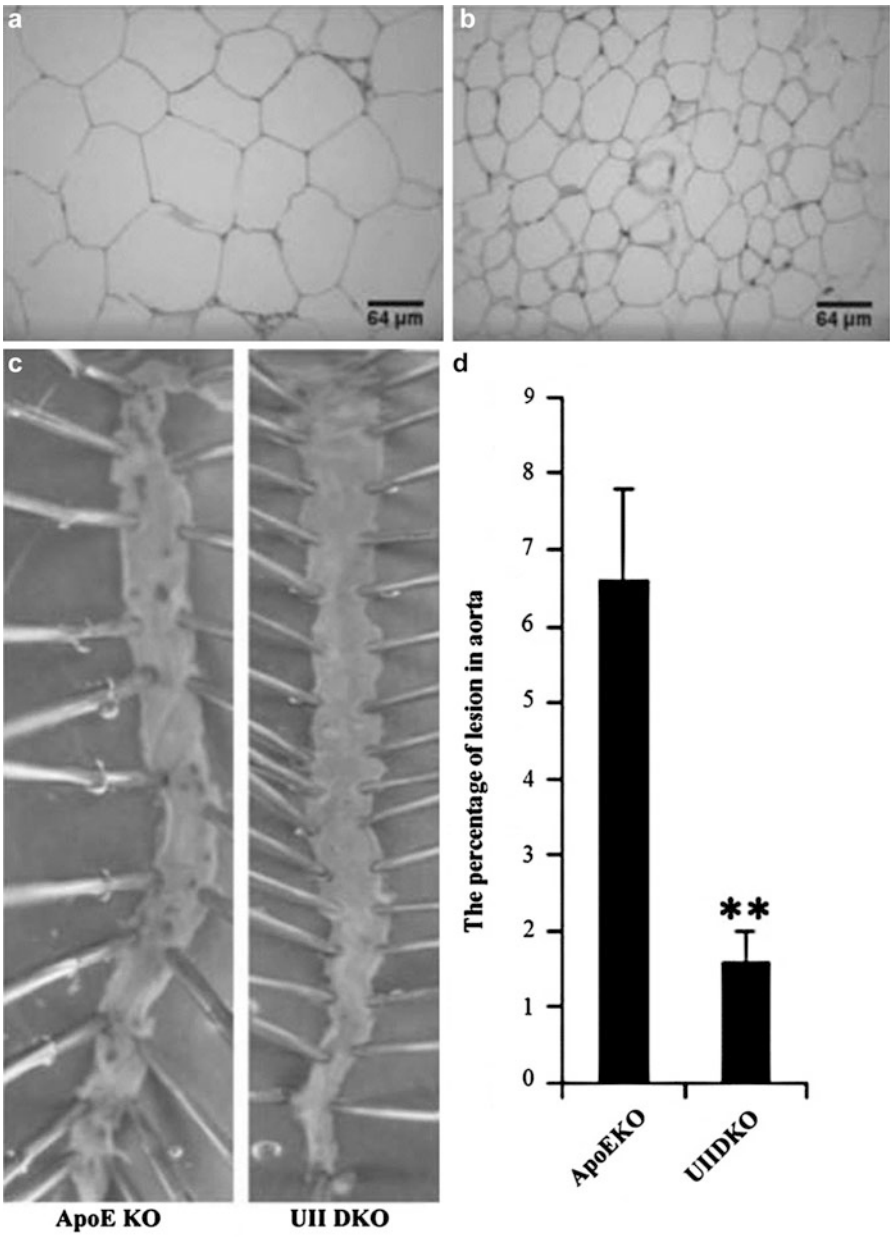

Fig. 5 Smaller adipocytes in visceral adipose tissue of UII KO mice compared to wild type mice (From You et al. (2012))

hypertrophy. Overexpression of UII and the UT receptor system in rat cardiomyocytes increases cardiomyocyte growth (Zou et al. 2001), enhances sarcomere organization (Zou et al. 2001), and produces a hypertrophic phenotype (Tzanidis et al. 2003). In addition to demonstrating UII-mediated hypertrophic effects, Johns et al. found that UII-stimulated cardiac myocytes secreted inflammatory cytokine IL-6, suggesting a potential pro-inflammatory role for UII in heart failure (Johns et al. 2004).

Future Research

Roles of the UT receptor system in cardiovascular disease are continuously emerging. There is significant evidence of the differential vasoactive effects of UII and in particular how endothelial cell dysfunction in pathophysiological conditions plays a key role in determining whether UII will mediate vasodilation or vasoconstrictive effects (Lim et al. 2004). As described previously, UII, URP, and UT levels have been found to be upregulated in various cardiovascular disease states, including but not limited to systemic and pulmonary hypertension, atherosclerosis, and congestive heart failure. Although there is abundant evidence linking the UT receptor system to cardiovascular disease, further research is required to develop a more complete understanding of the system's physiological and pathophysiological roles in the cardiovascular system. While several immunohistochemical analyses demonstrate increased UII expression in cardiovascular disease, little is known about the regulatory mechanisms underlying this process, such as the presence of urotensin converting enzymes (e.g., furin and trypsin) (Russell and Molenaar 2004) capable of converting prepro-UII into the mature peptide. However, the lack of specificity of these converting enzymes makes them less attractive pharmacological targets (Russell 2004). Recent studies using pharmacological inhibitors of the UT system are beginning to provide deeper insight into its specific roles in cardiovascular pathologies. Of particular interest is a recent study by Chatenet et al. where they have discovered two new UT receptor antagonists, [Pep(4)]URP and rUII(1–7), that are capable of distinguishing between UII and URP, providing a previously absent pharmacological method of distinguishing between UII and URP action in vitro and in vivo (Chatenet et al. 2013). Cardiovascular disease is a leading cause of death in North America. Determining the specific roles of UII and URP in the cardiovascular system and developing specific pharmacological agents are critical to achieving better insight into the pathology of these diseases as well as novel therapies (Figs. 4 and 5).

Summary Points

- This chapter focuses on vasoactive peptide urotensin II and its implication in cardiovascular physiology and pathophysiology.
- UII is a potent vasoconstrictor whose effects are dependent on species, vascular bed location, and endothelium status.

- The CNS localization of UII and its receptor as well as the elevated levels of this peptide seen in hypertension suggest a direct involvement of UII in the central regulation of blood pressure.
- Strong evidence suggests the direct involvement of UII in aortic atherosclerosis and many associated features such as intimal thickening, inflammation, and adipogenesis.
- Several studies have demonstrated elevated UII tissue and serum expression in CHF and a direct implication for this peptide in the associated hypertrophy.
- There is ample evidence linking the UII to cardiovascular disease, and further research is required to develop a more complete understanding of UII's physiological and pathophysiological roles in the cardiovascular system.

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Association of Fetuin-A with Carotid Intima-Media Thickness and Vascular Diseases

8

Aydın Akyüz

Contents

Abstract

Fetuin-A, also known as α2-Heremans–Schmid glycoprotein (AHSG), is a member of the cystatin superfamily of cysteine protease inhibitors and has different functions in human physiology and pathophysiology. Most studies suggest a biphasic effect of fetuin-A depending on the stages of atherosclerosis. Serum levels of fetuin-A are decreased in cases of acute inflammation. Therefore, it is known as a negative acute-phase protein. Fetuin-A inhibits insulin signaling and pathological calcification and has emerged as a diabetogenic agent. Fetuin-A levels are also found to be related to visceral obesity and dyslipidemia. Some authors have claimed that fetuin-A has a proatherogenic role, but it is still unclear whether high fetuin-A levels accelerate atherosclerosis except in the case of

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_16

diabetes mellitus (DM). One of the most important reasons for this uncertainty is the fact that there is a very weak compatibility between fetuin-A measurements performed by two different commercial enzyme-linked immunosorbent assay (ELISA) kits. Because the early sign of atherosclerosis is carotid intima-media thickness (CIMT), attention has been drawn to the relationship between CIMT and fetuin-A, especially in patients with DM. A number of studies in the literature have demonstrated an inverse correlation between fetuin-A and CIMT in patients who had chronic inflammatory disease but not DM. In addition, there is no association between fetuin-A and CIMT in subjects without known clinical cardiovascular disease. However, it seems that high fetuin-A levels accelerate atherosclerosis in DM and that diabetic patients exhibit a positive correlation between fetuin-A and CIMT.

Keywords

Fetuin-A • Carotid intima-media thickness • Arterial stiffness • Atherosclerosis • Inflammation

Abbreviations

AHSG	Alpha-2-Heremans–Schmid glycoprotein
AMI	Acute myocardial infarction
CAD	Coronary artery disease
CIMT	Carotid intima-media thickness
CRP	C-reactive protein
CVD	Cardiovascular disease
DM	Diabetes mellitus
ELISA	Enzyme-linked immunosorbent assay
HMGB1	High-mobility group protein-1
IFN	Interferon
IL	Interleukin
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
PAD	Peripheral artery disease
TGF-β	Transforming growth factor β
TNF	Tumor necrotizing factor

Key Facts About Fetuin-A

- Fetuin was obtained from bovine fetal serum for the first time in 1944. It is also called α2-Heremans–Schmid glycoprotein (AHSG).
- Fetuin-A is a glycoprotein synthesized from the liver and is found abundantly in the circulation. It is the main carrier protein in the fetal circulation and is found at a higher rate compared to albumin.
- Fetuin-A belongs to the cystatin superfamily. This family is known to comprise cysteine protease inhibitors, which are responsible for bone resorption.

- Fetuin-A is a glycoprotein synthesized predominantly from the liver and represents a great part of the α2 band of serum electrophoresis, with a molecular mass of approximately 60 KDa.

Key Facts About Fetuin-A Functionality

- Fetuin-A is known as a negative acute-phase protein. Proinflammatory cytokines (TNF, IL-1, IL-6, and IFN-γ) decrease the release of fetuin-A.
- Fetuin-A inhibits insulin receptor tyrosine kinase by binding to insulin receptor. Elevated fetuin-A levels lead to insulin resistance in muscle and adipose tissue and are associated with hypertriglyceridemia.
- Sialic acid residues of fetuin-A have the ability to bind to Ca++ ions. Fetuin-A binds to excessive calcium in the circulation and calciprotein particles are formed. Thus, it prevents calcification of soft tissues.

Key Facts About the Effects of Fetuin-A on CIMT

- Fetuin-A binds to excessive calcium in the circulation and calciprotein particles are formed. Thus, calcification of the soft tissues is prevented. Vascular calcification may be manifested through intimal or medial involvement.
- Intimal calcification generally occurs in atherosclerosis-related plaques and as a result of an inflammatory process related with cardiovascular risk factors including DM, hypertension, smoking, and dyslipidemia.
- Medial calcification usually occurs in patients with DM or patients receiving dialysis and generally progresses asymptomatically to a process called arteriosclerosis which leads to increased vessel stiffness.
- Carotid stiffness and CIMT are useful for determining the presence of atherosclerosis.
- All studies suggest a biphasic effect of fetuin-A depending on the stages of atherosclerosis.
- A number of studies in the literature have demonstrated an inverse correlation between fetuin-A and CIMT in patients with chronic inflammatory disease but not DM.
- There is no association between fetuin-A and CIMT in subjects without known CVD. However, it seems that high fetuin-A levels accelerate atherosclerosis in patients with DM who exhibit a positive correlation between fetuin-A and CIMT.

Definitions

Arterial stiffness Reduced capability of an artery to expand and contract in response to pressure changes due to loss of elastic fibers within the arterial wall

Atherosclerosis A condition where the arteries become narrowed due to plaque

Endotoxemia The presence of endotoxins in the blood

Fetuin-A A protein released by the liver and secreted into the bloodstream

A protective response of host cells, blood vessels, and proteins and other mediators to pathogens, damaged cells or irritants

Intima-media thickness A measurement of the thickness of the tunica intima and tunica media

N-linked glycosylation The attachment of glycan, a sugar molecule, to a nitrogen atom and amino acid residue in a protein

O-linked glycosylation The attachment of glycan, a sugar molecule, to an oxygen atom and amino acid residue in a protein

Phosphorylation The addition of a phosphate group to a protein

Sepsis A potentially fatal whole-body inflammation

Transforming growth factor β A protein that controls proliferation, cellular differentiation, and other functions in most cells

Introduction

Fetuin was first obtained from bovine fetal serum in 1944. It is also called α2-Heremans–Schmid glycoprotein (AHSG). Fetuin-A belongs to the cystatin superfamily. This family is known to comprise cysteine protease inhibitors, which are responsible for bone resorption. Fetuin-A is a glycoprotein that is synthesized predominantly from the liver; it is found abundantly in the circulation, with serum levels in the range of 0.4–1.0 g/L, and represents a great part of the α2-band of serum electrophoresis, with a molecular mass of approximately 60 KDa. Extrahepatic fetuin-A synthesis may occur in the kidney and the choroid plexus.

Fetuin-A expression is evident in all major organs during fetal development. It is the main carrier of protein in the fetal circulation and is found with a higher rate compared to albumin. This glycoprotein is cleared through binding to hepatocytes' asialo-glycoprotein receptor (Tolleshaug 1984) and through the formation of the protein–mineral complex, including fetuin, matrix gla protein, and calcium phosphate compounds (Price et al. 2002). This protein has several functions in human physiology and pathophysiology, including in bone metabolism, insulin resistance and diabetes mellitus (DM), ischemic stroke, and neurodegenerative diseases (Fig. 1). Although fetuin-A plays a role as a negative acute-phase reactant in

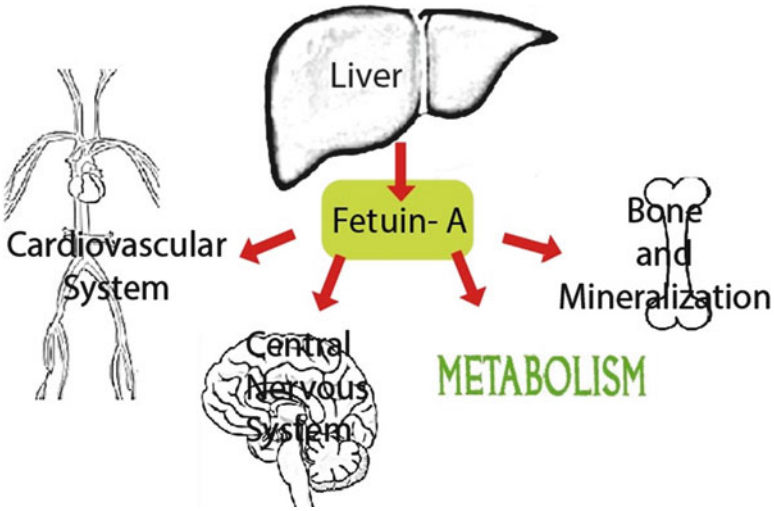

Fig. 1 Fetuin-A is secreted from the liver and has roles in bone mineralization, the cardiovascular and central nervous systems, and metabolism

subclinical atherosclerosis (Gangneux et al. 2003; Lebreton et al. 1979), its role in atherosclerosis is complex.

Fetuin-A exhibits protective effects against atherosclerosis through the inhibition of vascular calcification. However, it is also implicated in adipocyte dysfunction due to its inhibition of the insulin receptor, thereby seemingly promoting atherosclerosis (Ix and Sharma 2010). Elevated serum fetuin-A concentrations have been related to metabolic syndrome, obesity, type 2 DM, nonalcoholic fatty liver disease (Dogru et al. 2013), and events related to ischemic stroke (Tuttolomondo et al. 2010) and myocardial ischemia (Weikert et al. 2008). The potential functionality and prognostic value of fetuin-A in atherosclerosis are discussed in this review, especially in terms of its relationship with carotid intima-media thickness (CIMT), because of contradictory findings in the literature.

The Functionality of Fetuin-A

The serum levels of fetuin-A are decreased in cases of acute inflammation. Therefore, it is known as a negative acute-phase protein. Proinflammatory cytokines (tumor necrosis factor [TNF], interleukin 1 [IL]-1, IL-6, and interferon γ [IFN]-γ) decrease the release of fetuin-A (Lebreton et al. 1979; Gangneux et al. 2003; Daveau et al. 1988). However, Hennige et al. (2008) showed that fetuin-A strongly induced cytokine release in human monocytes in vitro and in mice in vivo; moreover, it had proinflammatory effects and suppressed atheroprotective adipokine adiponectin production. The other proinflammatory cytokine which determines this property is high-mobility group protein-1 (HMGB1). HMGB1 has been defined as a novel

proinflammatory cytokine and is released in the late phase in cases of endotoxemia and sepsis; moreover, in these disorders, fetuin-A is observed with a low level in the early phase and a high level in the late phase in blood (Li et al. 2011). A low level of fetuin-A has been found in conditions such as pancreatitis (Kusnierz-Cabala et al. 2010) and rheumatoid arthritis (Sato et al. 2007). Therefore, it has been defined as a negative acute-phase reactant.

Paradoxically, fetuin-A levels increase in cerebral ischemic injuries (stroke) (Weikert et al. 2008; Tuttolomondo et al. 2010). In traumatic injuries, it also increases, probably due to the release of HMGB1 protein (Zhu et al. 2010). In indirect injuries, it acts as a positive acute-phase protein. Therefore, it has a dual response to inflammation. Schure et al. reported that matrix metalloproteinases (MMPs), which are increased in inflammatory diseases such as periodontitis, bind and degrade fetuin and alter its ability to inhibit calcification in vitro; the increase in MMPs could affect the regulation of mineralization and potentially enhance the risk of the formation of calcified atheroma (Schure et al. 2013). In addition, fetuin-A binds to type 2 transforming growth factor β (TGF-β) receptors and competes with TGF-β (Demetriou et al. 1996). Fetuin-A carries two N-linked and three O-linked oligosaccharide chains ending with sialic acid residues. These sialic acid residues have the ability to bind to Ca++ ions (Fig. 2). Fetuin-A binds to excessive calcium in the circulation, resulting in the formation of calciprotein particles. Thus, the calcification of soft tissues is prevented (Jahnen-Dechent et al. 2011) (Fig. 3). Moreover, fetuin-A has been shown to prevent vascular calcium deposition, especially in animal models (Schafer et al. 2003). In addition, it mediates remodeling in bone formation by way of its inhibitory effect on TGF-β. This glycoprotein accumulates in the skeleton during mineralization and inhibits apatite formation due to its high binding affinity to hydroxyapatite (Schinke et al. 1996).

Fetuin-A is also involved in the release of insulin. Only two proteins can bind to the extracellular part of insulin receptors, namely, insulin and fetuin-A. In experimental models, it has been shown that fetuin-A inhibits insulin receptor tyrosine kinase by binding to insulin receptors (Auberger et al. 1989). Thus, increased fetuin-A levels lead to insulin resistance in muscle and adipose tissue (Rauth et al. 1992) and are associated with hypertriglyceridemia (Roos et al. 2010). Decreased plasma fetuin-A levels inhibit vascular calcification, while increased

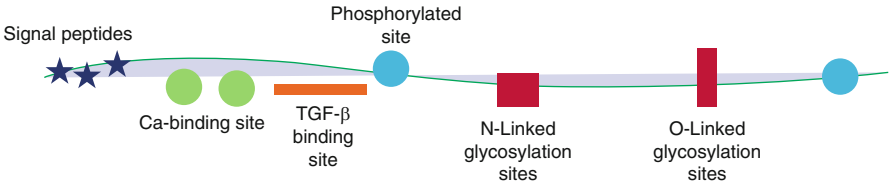

Fig. 2 The protein structure of fetuin-A has three carbohydrate units, which are present on a peptide chain linked with threonine and serine residues. Fetuin-A (AHSG) is a circulating serum glycoprotein with a molecular mass of approximately 60 KDa. There are N- and O-linked complexes in the structure, which may be responsible for the diverse functions of fetuin-A

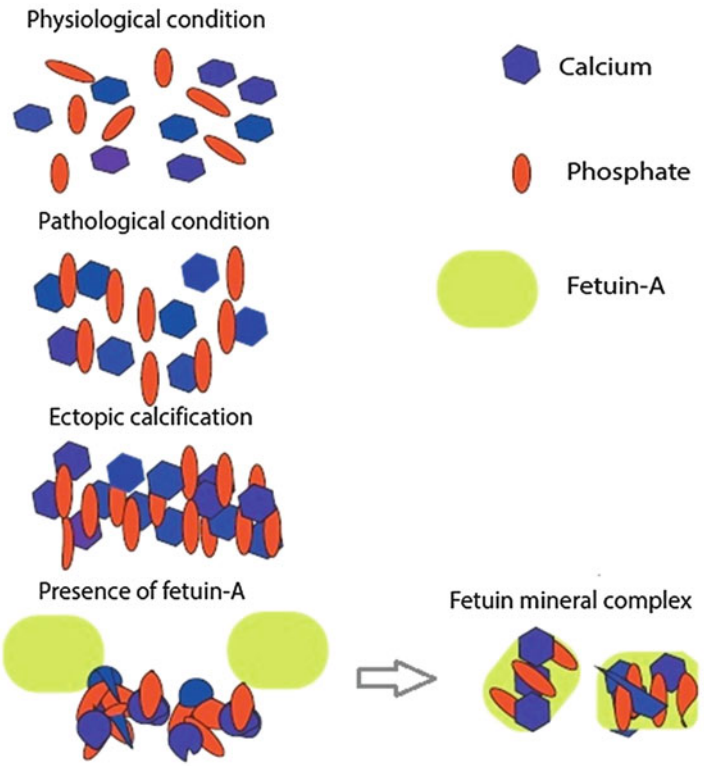

Fig. 3 Fetuin-A plays a role in calcium homeostasis and has an inhibitory effect on ectopic calcification. It is a potent inhibitor of spontaneous hydroxyapatite formation in supersaturated calcium- and phosphate-containing solutions. Low serum fetuin-A concentrations are associated with arterial calcification

serum fetuin-A levels may lead to insulin resistance and metabolic disorders (Ix et al. 2008; Stefan et al. 2008). Therefore, fetuin-A exhibits dual pathophysiological action. In addition, it has been reported that increased serum fetuin-A levels accelerate atherosclerosis by leading to insulin resistance (Fig. 4). Fetuin-A has been shown to be associated with acute myocardial infarction (AMI) and ischemic stroke (Weikert et al. 2008). Fetuin-A blood levels have been shown to be decreased, and vascular calcification has been observed at a high rate in patients with chronic renal failure on dialysis (Ketteler et al. 2003). In addition, low levels of fetuin-A in patients receiving dialysis have been shown to be related with increased mortality (Hermans et al. 2007a).

The presence of DM (Stefan et al. 2008), the level of renal functions (Mehrotra et al. 2005), obesity (Brix et al. 2010), the presence of obstructive sleep apnea (Akyuz et al. 2013), and blood levels of inflammatory cytokines (Gangneux et al. 2003; Lebreton et al. 1979) are the main confounding factors which determine

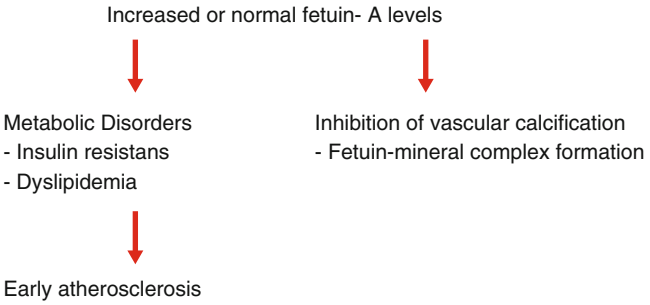

Fig. 4 Increased serum fetuin-A levels accelerate atherosclerosis by leading to insulin resistance, because it is a natural inhibitor of the insulin-stimulated insulin receptor tyrosine kinase. Increased fetuin-A levels are associated with obesity, metabolic syndrome, type 2 DM, and nonalcoholic fatty liver disease. Hyperglycemia and insulin resistance impair endothelium-derived nitric oxide production and promote early atherosclerosis. Fetuin-A inhibits hydroxyapatite formation

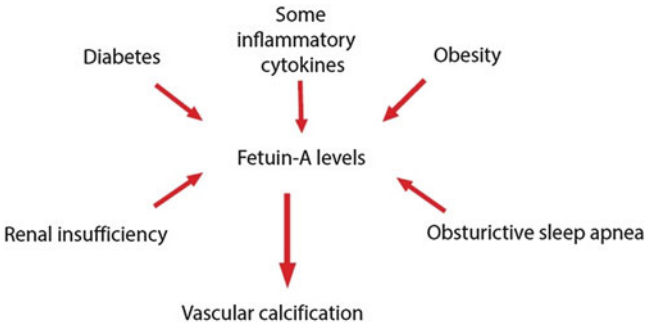

Fig. 5 The main confounding factors such as diabetes, obesity, renal insufficiency, obstructive sleep apnea, malnutrition, and some inflammatory cytokines can alter the serum levels of fetuin-A

the serum levels of fetuin-A (Figs. 5 and 6). Vascular calcification may be manifested as intimal or medial involvement (Ketteler et al. 2006). Intimal calcification generally occurs in atherosclerosis-related plaques and as a result of an inflammatory process related with cardiovascular risk factors, including DM, hypertension, smoking, and dyslipidemia. Medial calcification usually occurs in patients with DM or in patients receiving dialysis and generally progresses asymptomatically to a process called arteriosclerosis, which leads to increased vessel stiffness (Fig. 7). Although the publications in the literature have shown that medial calcification is related with fetuin-A deficiency, it is technically difficult to differentiate intimal and medial calcification, especially in patients with DM. In addition, it has recently been shown that other serum proteins, including matrix G1a protein, osteoprotegerin, and osteopontin, have an important role in the acceleration of vascular tissue calcification (Schlieper et al. 2007). Therefore, the effects of fetuin-A on inhibition of vascular calcification are also related with

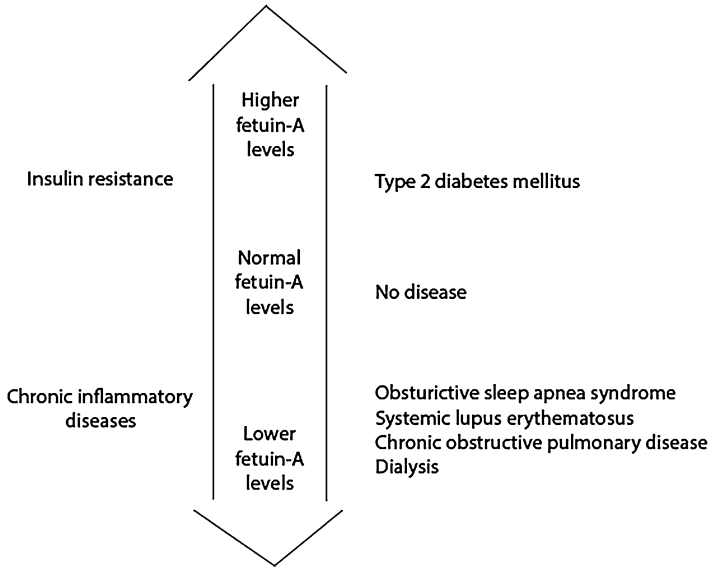

Fig. 6 Fetuin-A levels in relation to diseases

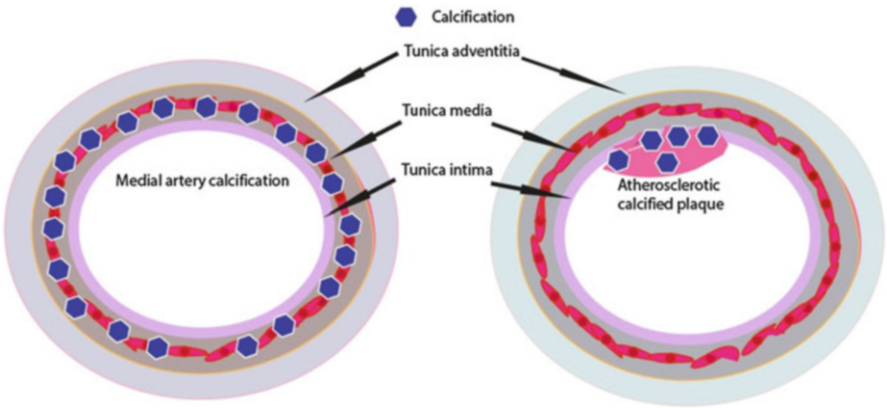

Fig. 7 There are two types of vascular calcification, namely, medial artery calcification and calcified intima atherosclerotic plaque (All figures were drawn by the author)

the serum levels of the serum proteins including matrix G1a protein, osteoprotegerin, and osteopontin (Kim et al. 2013; Schlieper et al. 2007). In addition, the measurement of serum fetuin–mineral complex rather than fetuin-A alone has been suggested a better marker of degree of extra-osseous calcification (Matsui et al. 2009).

Fetuin-A, Cardiovascular Disease, and CIMT

Many studies have shown that greater coronary artery calcification is associated with a greater risk of cardiovascular diseases (CVDs) such as angina pectoris, AMI, and stroke (Westenfeld et al. 2007; Mori et al. 2007; Folsom et al. 2008). There are disagreements about the role of fetuin-A in cardiovascular diseases, except in terms of the condition of vascular calcification in hemodialysis patients and in the early stage of atherosclerosis in subjects with normal renal function. Fetuin-A levels were significantly found to be decreased in patients with advanced three-vessel disease compared with those without stenosis and inversely correlated with advanced calcified coronary artery disease (CAD) in patients with normal renal function (Mori et al. 2010). However, it has also been reported that serum fetuin-A correlates positively with coronary artery calcification in nondialyzed diabetic patients with nephropathy (Mehrotra et al. 2005).

Another study (Mikami et al. 2008) suggested that there is no relationship between coronary artery calcification and serum fetuin-A levels. In addition, an inverse relationship has been found between mitral annular calcification and fetuin-A levels in patients with CAD but without uremia (Ziyrek et al. 2013). These data potentially demonstrate contradictory findings related to fetuin-A and CVD in the presence of DM and uremia. Decreased serum concentrations of the calcification inhibitor fetuin-A are related to increased cardiovascular mortality in dialysis patients. Although fetuin-A-deficient rats have various soft tissue calcifications rather than vasculature due to the protection of the intact endothelium without atherosclerosis, fetuin-A deficiency accelerates intimal rather than medial calcification of atherosclerotic plaques.

Fetuin-A inhibits pathological calcification in both the soft tissue and vasculature, even in the setting of atherosclerosis (Westenfeld et al. 2009). Arterial calcification is evident in the medial or intimal vascular layer. Intimal layer involvement is the main characteristic of atherosclerosis. Calcification caused by fetuin-A deficiency usually occurs in the medium- and large-sized arteries, myocardium, or heart valves. Medial layer calcifications usually occur within the lamina elastica interna and smooth muscle cell layer. However, one study put forward that fetuin-A levels are increased in patients with type 2 DM and peripheral arterial disease (PAD) (Lorant et al. 2011), and it is inversely associated with medial sclerosis; meanwhile, another study demonstrated that lower circulating fetuin-A is associated with PAD in type 2 DM (Eraso et al. 2010). Szeberin et al. showed that fetuin-A levels are negatively associated with the severity of atherosclerosis in nonuremic patients with PAD due to its putative protective role in the progression of vessel calcification (Szeberin et al. 2011). In other words, there are contradictory results concerning the relationship between fetuin-A and PAD.

Interestingly, PAD patients with medial sclerosis have lower serum fetuin-A concentrations compared to those without medial sclerosis. According to these findings, fetuin-A has dual effects on vascular atherosclerosis, both as an atherogenic factor and a calcification inhibiting factor. It is possible that fetuin-A levels might change according to the balance between the severity of arterial wall calcification

and atherosclerosis progression. In addition, fetuin-A might increase the collagen content of the arterial wall by blocking TGF-β signaling, thereby accelerating arterial stiffness.

Fetuin-A-deficient rats have normal phenotype but exhibit severe calcification of various organs (Westenfeld et al. 2009). Increased organ calcification in the heart or kidney may accelerate myocardial or renal dysfunction (Schafer et al. 2003). Intramyocardial calcification with fibrotic tissue is associated with diastolic dysfunction, less ischemic tolerance, and decreased sympathetic response. Both the inverse relationship between fetuin-A levels and coronary artery calcification in patients with renal disease (Westenfeld et al. 2007) and the positive relationship between fetuin-A levels and peripheral artery calcification in nondialyzed diabetic patients with renal dysfunction (Mehrotra et al. 2005) potentially suggest that fetuin-A counteracts vascular calcification in the early stages of DM and atherosclerosis. Some studies showed that dyslipidemia and hyperinsulinemia increase secretion of hepatic fetuin-A (Ix et al. 2006; Stefan et al. 2006). One study reported that serum fetuin-A levels are positively related to the degree of atherosclerosis (Rittig et al. 2009). Another showed that high serum fetuin-A levels are correlated with increased cardiovascular risk, irrespective of the presence of DM (Weikert et al. 2008). Increased fetuin-A levels might facilitate both atherosclerotic progression and insulin resistance (Rittig et al. 2009).

Some studies have shown that there is an inverse correlation between fetuin-A and adiponectin levels in patients with increased cardiovascular risk (Hennige et al. 2008; Ix and Sharma 2010). Decreased serum adiponectin levels might increase serum-free fatty acid levels, thereby causing atherosclerosis or accelerating atherosclerotic progression. Given these convincing findings, the modulation of adiponectin caused by fetuin-A appears to be an important factor in atherosclerosis progression (Hennige et al. 2008; Ix and Sharma 2010). Mori et al. found (2007) a positive relationship between fetuin-A and arterial stiffness, an important marker of atherosclerosis, in healthy subjects. Moreover, Fiore et al. reported that fetuin-A levels are positively correlated with arterial intima-media thickness, an indicator of remodeling of the arterial wall (Fiore et al. 2007). A study by Merx et al. was the first to show the functional role of isolated myocardial calcification independent of arterial stiffness in fetuin-A-deficient mice and found impaired left ventricle relaxation due to dystrophic cardiac calcification. Remarkably, these researchers also identified an association with the profound induction of profibrotic TGF-β and downstream collagen and fibronectin mRNA in these mice (Merx et al. 2005). Merx et al. also suggested that higher serum fetuin-A levels might be protective for some cardiovascular diseases, because of fetuin-A's ability to prevent calcium/phosphate precipitation and ectopic mineralization in the arterial wall (Merx et al. 2005).

Lower fetuin-A levels are related to higher inflammatory response and cause the release of some cardiotoxic cytokines (e.g., TNF) (Ombrellino et al. 2001), and the inverse relationship between cardiotoxic cytokines and cardiac contractility has been well documented (Kelly and Smith 1997). In addition, a negative relationship between serum C-reactive protein (CRP) and fetuin-A levels was found to be decreased in patients with CAD (Bilgir et al. 2010), as well as in dialysis patients

(Hermans et al. 2007a). CRP is an important acute-phase inflammatory protein caused by IL-6 secretion from macrophages.

At present, increased serum CRP levels are used for determining cardiovascular risk (Danesh et al. 2004). Interestingly, Kadaglou et al. showed that statin therapy reduces fetuin-A levels, as well as serum total cholesterol, low-density lipoprotein cholesterol, and CRP levels (Kadoglou et al. 2014). Zhao et al. documented that serum fetuin-A levels are related to the presence and severity of CAD in DM patients and put forward that fetuin-A might be used as a marker for the progression of CAD in patients with DM (Zhao et al. 2013). Elevated fetuin-A levels were a negative predictor of CAD and an independent predictor of nonalcoholic fatty liver disease (Ballestri et al. 2013). Afsar et al. found lower serum fetuin-A levels in patients with acute coronary syndrome, independent of heart valve calcification, and defined fetuin-A as a negative acute-phase protein after AMI (Afsar et al. 2012). In addition, a fetuin-A level lower than 140 mg/L was shown to be a predictor of death at 6 months after ST-elevation AMI (Lim et al. 2007). Plasma fetuin-A levels usually decrease within a few hours after the onset of AMI and reach normal serum levels in 5–7 days (Mathews et al. 2002). Roos et al. demonstrated that serum fetuin-A levels did not predict cardiovascular events during 6 years of follow-up in 1,049 patients (Roos et al. 2010).

CIMT and Atherosclerosis

Age, hyperlipidemia, hypertension, DM, smoking, and sedentary lifestyle are the factors which increase CIMT. Measurement of CIMT by ultrasonography is an inexpensive, simple, reliable, and reproducible noninvasive method. CIMT can also be shown by magnetic resonance imaging, but its measurement by this method is not recommended. The thickness of the tunica intima and tunica media, which constitute the inner layer of the arterial wall, is measured by ultrasonography. In addition, ultrasonography is also useful for determining the presence of atherosclerosis (Baldassarre et al. 2012) and the efficiency of lipid lowering (Hodis et al. 1996) or antihypertensive drug usage (Pitt et al. 2000). However, in recent meta-analyses, it has been recommended only as an assistive method in determining cardiovascular risk, not for direct risk assessment (Costanza et al. 2010; Lorenz et al. 2012; Den Ruijter et al. 2012). Nevertheless, the American Heart Society and American College of Cardiology recommended measuring CIMT to obtain a better risk assessment in asymptomatic patients with moderate cardiovascular risk (Goff et al. 2014). Measurement of CIMT is not recommended for patients with low or high risk or for patients with known cardiovascular disease. CIMT measurements are performed on the posterior carotid artery just above the bulbus from the area which does not contain plaque (Montauban van Swijndregt et al. 1999). Normal CIMT is approximately 0.4–0.5 mm at the age of about 10 years, while it is approximately 0.7–0.8 mm in adulthood. In adults, a value $\geq$0.9 mm is considered high. Localized thickenings of at least 1.5 mm and above are considered plaque.

Fetuin-A and CIMT

In the literature, it is still unclear whether the relation between fetuin-A and carotid stiffness and CIMT is positive or negative. However, there are studies showing that fetuin-A is a negative inflammatory marker (Gangneux et al. 2003) and inversely correlated with aortic (Roos et al. 2009) and carotid stiffness (Akyuz et al. 2013) in patients with chronic inflammatory diseases in contrast to the publication's relation with diabetic patients. Guarneri et al. (2013) found that CIMT was inversely correlated with fetuin-A in patients with essential hypertension. In a study we performed (Akyuz et al. 2013), an inverse correlation was found between fetuin-A and CIMT in normotensive patients with obstructive sleep apnea. Mori et al. demonstrated that fetuin-A levels are significantly associated with carotid artery stiffness in healthy subjects (Mori et al. 2007), but they did not study the associate fetuin-A with CIMT. The above mentioned studies suggest a biphasic effect of fetuin-A depending on the stages of atherosclerosis.

The Positive Correlation Between Fetuin-A and CIMT in Patients with DM

It is thought that fetuin-A is metabolically related with the initiation and progression of atherosclerosis, like DM, by triggering insulin resistance in the muscle and adipose tissue. Studies have shown a positive correlation between fetuin-A levels and increased CIMT and carotid stiffness (Mori et al. 2007), especially in patients with type 2 DM (Dogru et al. 2013; Fiore et al. 2007; Rittig et al. 2009; Yin et al. 2014; Koluman et al. 2013) or insulin resistance (Dogru et al. 2013) (Table 1). In addition, serum fetuin-A levels have been reversely correlated with carotid and femoral artery calcifications in patients with type 2 DM with preserved renal function (Emoto et al. 2010). In these studies, fetuin-A has been reported to lead to increased CIMT or increased stiffness because of its diabetogenic effect and proinflammatory properties. These studies mostly suggest that fetuin-A levels might represent a surrogate marker for the severity of the atherosclerosis in patients with type 2 DM and increased CIMT.

The Negative Correlation Between Fetuin-A and CIMT in Patients with Chronic Inflammatory Disease

A number of studies in the literature have demonstrated an inverse correlation between fetuin-A and CIMT in patients with chronic inflammatory disease but not DM. According to the findings of these studies, since fetuin-A is a negative acute-phase reactant, low serum fetuin-A concentrations could be a consequence of the chronic inflammatory state in conditions such as chronic obstructive pulmonary disease (COPD) (Alpsoy et al. 2014), obstructive sleep apnea (Akyuz et al. 2013), uremia (Hermans et al. 2007b; Wang et al. 2007), systemic lupus erythematosus (Abdel-Wahab et al. 2013) or subclinical vascular inflammation caused by essential hypertension (Guarneri et al. 2013).

Table 1 Studies demonstrating whether there is a positive, negative or no correlation between fetuin-A and CIMT

Patients/subjects	The correlation between fetuin-A and CIMT	Fetuin-A analysis	References
New-onset type 2 DM ($n = 100$)	Positively	ELISA kit (R&D Systems, Minneapolis, MN, USA)	Yin et al. (2014)
Type 2 DM ($n = 120$)	Positively (only in normoalbumineric diabetic patients)	ELISA kit (BioVendor Human Elisa kit, Brno, Czech Republic)	Koluman et al. (2013)
The subjects at risk for type 2 DM ($n = 315$)	Positively	–	Rittig et al. (2009)
With NAFLD and insulin resistance ($n = 115$)	Positively	ELISA kit (Epitope Diagnostics, Inc., San Diego, USA)	Dogru et al. (2013)
Peripheral artery disease with low bone mass ($n = 90$)	Positively (51 % patients with DM)	ELISA (Human Fetuin ELISA Kit, Epitope Diagnostics Inc., San Diego, CA, USA)	Fiore et al. (2007)
Dialysis patients ($n = 134$)	Negatively	Nephelometry method	Hermans et al. 2007b
Dialysis patients ($n = 147$)	Negatively	ELISA kit (Epitope Diagnostics, Inc., San Diego, USA)	Wang et al. (2007)
Systemic lupus erythematosus ($n = 40$)	Negatively	ELISA kit (Epitope Diagnostics, Inc., San Diego, USA)	Abdel-Wahab et al. (2013)
Without cardiovascular disease ($n = 1374$)	No	ELISA kit (Epitope Diagnostics, Inc., San Diego, USA)	Ix et al. (2011)
Normotensive chronic obstructive pulmonary disease ($n = 65$)	Negatively	ELISA kit (BioVendor Human Elisa kit, Brno, Czech Republic)	Alpsoy et al. (2014)
Normotensive obstructive sleep apnea syndrome ($n = 50$)	Negatively	ELISA kit (BioVendor Human Elisa kit, Brno, Czech Republic)	Akyuz et al. (2013)

CIMT carotid intima-media thickness, *DM* diabetes mellitus, *NAFLD* nonalcoholic fatty liver disease

The Lack of Correlation Between Fetuin-A and CIMT in Subjects Without Known CVD

Ix et al. demonstrated that there was no association between fetuin-A and CIMT in a large population ($n = 1,375$) without known clinical CVD; here, fetuin-A was only inversely correlated with severity of carotid artery calcification (Ix et al. 2011). In addition, a correlation was found between fetuin-A and carotid stiffness, while no correlation was found between fetuin-A and CIMT in a study involving healthy subjects performed by Mori et al. (2007).

The Reasons for the Uncertain Fetuin-A Results in the Literature

It is still unclear whether high fetuin-A levels accelerate atherosclerosis, except in the case of DM. One of the most important reasons for this uncertainty is the fact that there is a very weak compatibility between fetuin-A measurements performed by two different commercial enzyme-linked immunosorbent assay (ELISA) kits (BioVendor Research and Diagnostic Products vs. Epitope Diagnostics, Inc.) (Smith et al. 2010). In the ELISA tests, specific antibody responses to different glycosylated forms of fetuin may be variable. Nephelometry is also used for measurement of fetuin-A. Therefore, fetuin-A measurements should be standardized. In addition, the companies which manufacture these kits still have not reported the normal values in healthy individuals. The other reason is that some threonine and serine residues of fetuin-A are modified with N-linked and O-linked glycosylation and phosphorylation. In this case, fetuin-A may have different functional properties (Gejyo et al. 1983; Yoshioka et al. 1986). Therefore, the levels of modified fetuin-A should also be determined in clinical studies. Thus, more studies are needed to determine the role of fetuin-A in determining CIMT and carotid artery stiffness.

Potential Applications to Prognosis and Other Diseases or Conditions

Fetuin-A has roles in bone metabolism, insulin resistance and DM, ischemic stroke, and neurodegenerative diseases. Some data suggest a link between high plasma fetuin-A levels and increased AMI and ischemic stroke. Low levels of fetuin-A, a systemic calcification inhibitor, are linked to mortality in patients on dialysis. One study suggested that a fetuin-A level lower than 140 mg/L is a predictor of death at 6 months after ST-elevation AMI (Lim et al. 2007). However, there are no exact data concerning fetuin-A for potential applications to prognosis, except in the case of chronic renal disease.

Summary Points

- This chapter focuses on the relationship between fetuin-A and CIMT.
- Fetuin-A has several functions in human physiology and pathophysiology, including in bone metabolism, insulin resistance and DM, ischemic stroke, and neurodegenerative diseases. The serum levels of fetuin-A are decreased in cases of acute inflammation. Therefore, it is known as a negative acute-phase protein. Fetuin-A also prevents calcification of soft tissues, especially in the vascular system.
- The companies which manufacture fetuin-A kits have still not reported the normal values in healthy individuals.

- There are no exact data concerning fetuin-A for potential applications to prognosis, except for chronic renal disease. Low fetuin-A is associated with increased mortality in dialyzed patients.
- Age, hyperlipidemia, hypertension, DM, smoking, and sedentary lifestyle are the factors which increase CIMT.
- Although increased CIMT is accepted as an early marker of atherosclerosis, its measurement is only recommended for a better risk assessment in asymptomatic patients with a moderate cardiovascular risk.
- A number of studies in the literature have demonstrated an inverse correlation between fetuin-A and CIMT in patients with chronic inflammatory disease and without DM. There is no association between fetuin-A and CIMT in subjects without known clinical cardiovascular disease. However, it seems that high fetuin-A levels accelerate atherosclerosis in DM and diabetic patients exhibit a positive correlation between fetuin-A and CIMT.
- It is still unclear whether high fetuin-A levels accelerate atherosclerosis, except in the case of DM. One of the most important reasons for this uncertainty is the fact that there is very weak compatibility between fetuin-A measurements performed by two different commercial ELISA kits. In addition, nephelometry is used for the measurement of fetuin-A. Therefore, fetuin-A measurements should be standardized. Some threonine and serine residues of fetuin-A are modified with N-linked and O-linked glycosylation and phosphorylation. In this case, fetuin-A may have different functional properties.

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Serum Cholinesterase Activities as Biomarkers of Cardiac Malfunctioning

9

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Contents

Abstract

Cardiovascular functioning depends on proper autonomous nervous system activities, and cardiovascular pathologies are associated with autonomic imbalance. Acetylcholine is the main parasympathetic neurotransmitter, involved in restoring hemostasis, reducing heart rate, and blocking inflammation and anxiety. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), the acetylcholine-hydrolyzing enzymes, can be detected via reliable, low-cost measurements that are amenable for use in large

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_10

cohort studies. Recent surveys have suggested that cholinesterase (ChE) activity measurements can serve as biomarkers for cardiovascular diseases, correlating with inflammation, heart rate, and delayed heart rate recovery. Moreover, patients with ChE activities beyond the normal range are at risk for non-survival or poor recovery outcome following stroke or myocardial infarction. In this chapter, we will introduce the ChEs, the detection methods available for measuring their activities, and the relevant studies validating the roles of ChEs as risk factors for cardiovascular diseases.

Keywords

AChE • BChE • Cholinesterases • Coronary arterial disease • Nanoparticles • Parasympathetic • MACE • Myocardial infarction

Abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
ANS	Autonomous nervous system
ATCh	Acetylthiocholine
BChE	Butyrylcholinesterase
BRS	Baroreflex sensitivity
BTCh	Butyrylthiocholine
CAD	Coronary arterial disease
ChAT	Choline acetyltransferase
CHD	Coronary heart disease
ChE	Cholinesterase
ChOx	Choline oxidase
CNS	Central nerve system
ColQ	Collagen tail
CS	Cholinergic status
DTNB	5,5′-Dithiobis-(2-nitrobenzoic acid)
GPI	Glycosylphosphatidylinositol
Il	Interleukin
iso-OMPA	Tetramonoisopropyl pyrophosphortetramide
MACE	Major adverse cardiac events
mAChR	Muscarinic ACh receptor
MI	Myocardial infarction
MiRNA	MicroRNA
nAChR	Nicotinic ACh receptor
NMJ	Neuromuscular junction
NP	Nanoparticle
PAS	Peripheral anionic site
PNS	Peripheral nervous system
PRiMA	Proline-rich membrane anchor
SNP	Single nucleotide polymorphism
TNF-α	Tumor necrosis factor-α
WAT	Tryptophan-rich amphiphilic tetramerization

Key Facts of Serum Cholinesterases

- The acetylcholine-hydrolyzing cholinesterase enzymes control the termination of cholinergic signaling in multiple tissues and are targets for a variety of commonly used drugs.
- The cholinesterases, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), differ on their substrate specificities and their sensitivities to selective inhibitors.
- The cholinesterases are modulated under inflammatory insults, affecting the cholinergic anti-inflammatory pathway.
- BChE is the major ACh-hydrolyzing enzyme in the circulatory system, but AChE hydrolyzes acetylcholine 20-fold faster than BChE.
- Modified serum cholinesterase activities have been implicated in stress-related diseases, stroke, metabolic syndrome, diabetes, and myocardial infarction.

Introduction

Coronary arterial disease (CAD) and its main complication, myocardial infarction (MI), is the leading cause of death worldwide (Deloukas et al. 2013). Traditional risk factors (such as hypertension, diabetes, plasma lipid concentrations, or biomarkers of inflammation) and lifestyle factors (such as smoking, obesity, and physical inactivity) are notably associated with coronary heart disease (CHD) risk and MI. Though the causes of cardiovascular disease are diverse, atherosclerosis is considered to be the most common one. Atherosclerosis is a chronic inflammation of arteries, which develops over decades in response to the biological effects of risk factors (Ross 1986).

Atherogenesis begins with endothelial dysfunction, causing endothelial and smooth muscle cells to proliferate and produce extracellular matrix molecules, eventually forming a fibrous cap. Plaques lead to clinical symptoms by producing flow-limiting stenosis or by provoking thrombi that interrupt blood flow on either a temporary basis (causing unstable angina) or a permanent one (causing MI) (Nabel and Braunwald 2012).

Ample clinical and experimental data suggests that cardiovascular functioning is tightly linked to autonomous nervous system (ANS) activities. Autonomic imbalance, being either a decrease in vagal tone or an increase in sympathetic activity is associated with increased mortality due to cardiovascular pathologies including MI (Schwartz et al. 1992) and cardiac arrhythmias (La Rovere et al. 2001). Indirect measures of cardiac parasympathetic dysfunction (such as elevated resting heart rate, delayed heart rate recovery from exercise, and attenuated heart rate increase during exercise) have also been shown to be predictors of adverse cardiovascular outcome (Jouven et al. 2005; Leeper et al. 2007). Along these lines, this chapter is devoted to the issue of circulation cholinesterase activities as potential biomarkers and predictors of cardiovascular impairments.

Acetylcholine (ACh) is the principal neurotransmitter of the parasympathetic branch of the ANS, and its levels are primarily regulated by the closely related enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). The classical role of the cholinesterases (ChEs) in terminating ACh-mediated neurotransmission, is now known as only one part of the complex set of roles of these enzymes, revealing nonclassical functions in neuritogenesis, cell adhesion, hematopoiesis, thrombopoiesis, and the regulation of pro-inflammatory agents (Darvesh et al. 2003; Soreq and Seidman 2001). In this chapter, we will present the cholinesterases, discuss their functions in health and disease, and review the current evidence for the value of cholinesterase activity measurements in body fluids as potential biomarkers for assessing cardiac function and pathology and estimating risks of cardiac disease.

AChE and BChE: The Cholinesterase Enzymes

ACh was the first neurotransmitter to be identified, already in 1914 by Henry Hallett Dale and Otto Loewi ("The Nobel Prize in Physiology or Medicine 1936." Nobelprize.org. Nobel Media AB 2014). Soon after, the existence of enzymes responsible for ACh hydrolysis has been validated, and these enzymes were given the general name cholinesterases. By the early 1950s, Mendel and Rudney first introduced functional definitions that divided the ChEs into two distinct types – specific and "pseudo" cholinesterase, today known as AChE and BChE, respectively.

The cholinesterases are serine hydrolases, belonging to the alpha/beta hydrolase fold superfamily. They share high sequence homology and present a 3D structural resemblance with a catalytic triad, glutamic acid, histidine, and serine, deeply embedded at the bottom of a narrow cavity, known as the active site "gorge" (Fig. 1a, left). However, as suggested by their initial names, they differ by their hydrolysis efficiency and substrate specificity. AChE primarily hydrolyzes ACh and presents narrow substrate specificity, whereas BChE can hydrolyze a broader range of substrates but shows 20-fold less efficiency for ACh hydrolysis (Darvesh et al. 2003; Soreq and Seidman 2001).

The catalytic differences between AChE and BChE primarily reflect distinct structural and amphipathic features of their active site gorges. The active site gorge in all ChEs includes the catalytic triad that hydrolyzes substrates by a "charge relay" mechanism and sub-domains located proximal to the middle of the gorge which orient the substrates with respect to the catalytic triad (Dvir et al. 2010). These include the anionic choline-binding site that interacts with substrates' cationic groups and the acyl pocket which accommodates the substrates' acyl groups. Another sub-domain, the "oxyanion hole," is important for stabilizing the transition state acquired by the substrate during hydrolysis. In addition, lining the gorge are aromatic amino acids, 14 in AChE and 8 in BChE. This difference and the corresponding bulk differences in the volume of these amino acid residues enlarge the volume of the active site gorge of BChE by approximately 200Å^3 in comparison

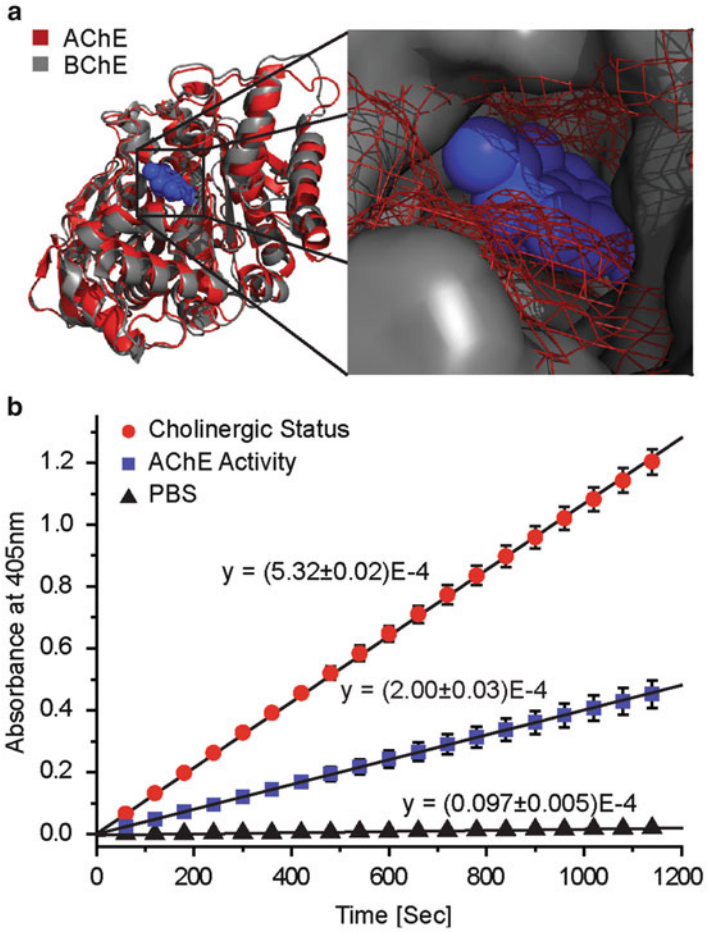

Fig. 1 Cholinesterase enzymes. (**a**) AChE (*red*) and BChE (*gray*) share high structure homology (*left*). Modeling of galantamine in the active site gorge of AChE (*red*) and BChE (*gray*) resemblance the differences in the active site volume (*right*). AChE's surface in the active site gorge stands out from that of BChE interfering to the access of large substrates. (**b**) AChE's contribution to the CS (total ACh hydrolysis capacity) demonstrated by Ellman's assay. The contribution of BChE can be calculated by reducing AChE activity contribution to the cholinergic status. Figure (**b**) follows Arbel et al. (2014)

to AChE; consequently, the distinct volume affects the access and orientation of molecules entering the active site gorge (Fig. 1a, right) (Nicolet et al. 2003).

A second major domain that is responsible for the differences between ChEs is the "peripheral anionic site" (PAS), a secondary substrate-binding site residing on the protein surface, in proximity to the entrance of the gorge. This site interacts with some of the substrates and inhibitors of the enzymes, is responsible for some of the non-hydrolytic functions of the ChEs, and can allosterically modulate their catalytic

activity (Johnson and Moore 2003; Bourne et al. 2003). Differences in the ChE's PAS, for example, the number of aromatic residues in it, are implicated with the type of allosteric modulation, substrate inhibition or substrate activation, in the presence of excess ACh, for AChE and BChE, respectively (Masson et al. 2001; Radic et al. 1991).

Cholinesterase Isoforms

The diversity of ChEs extends even further due to common single nucleotide polymorphisms (SNPs) and rare mutations in the ChE genes, as well as due to epigenetic, posttranscriptional, and posttranslational modifications. The human *ACHE* and *BCHE* genes are located in two chromosomal separate loci: AChE at 7q22, with six exons and four introns, and BChE at 3q26, with four exons and three introns. So far, 19 SNPs were identified for *ACHE* and 40 for *BCHE*, some of them with significant effects on ChE interactions with substrates, inhibitors, and other proteins (Hasin et al. 2005; Gnatt et al. 1994; Howard et al. 2010; Darvesh et al. 2003).

The genomic regions harboring the ChE genes include complex promoter structures that enable selective expression under specific conditions. In particular, the AChE promoter allows the inclusion of diverse N-terminal peptides in the mature AChE protein (Meshorer et al. 2004, 2005). Pre-transcriptional changes in these ChE genomic domains involve epigenetic changes in their extended promoter regions, for example, excessive and long-lasting histone acetylation of the AChE promoter under psychological stress (Sailaja et al. 2012). Posttranscriptional modifications mainly involve alternative splicing of the AChE pre-mRNA but not BChE pre-mRNA. These contribute to the complexity of ChEs by producing important AChE isoforms with different N- and/or C-terminal domains that determine their subcellular localization and biological functions. At the 3′ end, this yields the membrane-attached AChE-S (also known as AChE-T for "tailed"), the glycosylphosphatidylinositol (GPI)- tethered AChE-E (also known as H for "hydrophobic"), and the soluble monomeric AChE-R ("readthrough") variants. AChE-S and AChE-E are the major isoforms expressed under normal conditions, resulting from splicing out or inclusion of exon 5, whereas AChE-R is expressed under various stressful insults and is encoded by mRNA which contains the coding pseudointron 4. Alternative 5′-end domains of AChE mRNA result from alternate promoter usage, also increasing the plethora of AChE isoforms by the possible translation of AChE with an extended N-terminus (N-AChE) (Meshorer et al. 2004); the combination of N-extended AChE with the C-terminal peptide of the AChE-S isoform is lethal to many cell types (Toiber et al. 2008). N-AChE is anchored to the plasma membrane of blood cells (Meshorer et al. 2004), suggesting that N-AChE-R may likewise be anchored to the synaptic structures.

All of the molecular forms of AChE possess identical core structures and present similar catalytic properties. However, the translation of the corresponding mRNAs results in different C-termini that have dramatic effects on the localization, oligomerization, protein-protein interactions, and biological functions of the AChE variants (Soreq and Seidman 2001). The AChE-S C-terminus is extended by 40 amino

acids in comparison to the AChE-R sequence, whereas the AChE-E C-terminus is extended only by 14 amino acids due to additional posttranslational cleavage. These extended C-termini contain cysteine residues that allow dimerization of the AChE-E and AChE-S variants. These dimers can later form tetramers stabilized by hydrophobic interactions between their characteristic tryptophan-rich amphiphilic tetramerization domains (WAT). Furthermore, the latter tetramers can form multimeric complexes by anchoring to the cell membrane through partner proteins including proline-rich domains, such as the proline-rich membrane anchor (PRiMA) in the central nervous system (CNS) (Noureddine et al. 2008), the collagen tail (ColQ) at the neuromuscular junction (NMJ) (Engel 2012), or the proline-rich peptides derived from lamellipodin in serum (Li et al. 2008). BChE as well can form dimers through disulfide bonds that later extend into tetramers and multimers through their C-terminal domains. Hence, AChE-R is the only known ChE that maintains the form of soluble monomers. The significance of this difference is emphasized by the dire consequences of inherited interferences in the interaction of AChE with ColQ. For example, congenital myasthenia results from mutations in the AChE binding sequence of ColQ (Engel 2012).

In addition to the oligomerization and proteolytic processing events mentioned above, the ChEs are subject to posttranslational modifications that affect their properties and functions. Those include attachment of glycophosphoinositide (GPI) to the C-terminus of AChE-E, which allows its anchoring to erythrocyte membranes. Glycosylation of AChE and BChE was further found to affect their membrane trafficking and stability with altered glycosylation patterning observed in Alzheimer's disease patients (Saez-Valero et al. 2003).

Cholinesterase Functions in Healthy Tissues

ACh release to the synaptic cleft and its binding in postsynaptic cells to the plethora of neuronal and/or nonneuronal nicotinic ACh receptors (nAChR), ligand-gated ion channels, and to the set of superfamily members of the G-protein-coupled muscarinic ACh receptors (metabotropic, mAChR) initiate well-known cascades of biochemical and electrophysiological responses as summarized in Soreq and Seidman (2001). Hence, the capacity of ChEs to hydrolyze ACh is one of the main mechanisms for regulating ACh-mediated neurotransmission at the NMJ, CNS, and peripheral nervous system (PNS).

Recent findings established the capacity of the cholinergic system not only to handle the termination of ACh neurotransmission but also to restore homoeostasis. For example, the cholinergic anti-inflammatory reflex inhibits NF-κB-mediated cytokine synthesis and release (Borovikova et al. 2000; Ofek et al. 2007). Both in the brain and in the periphery, ACh shows anti-inflammatory properties that inhibit innate immune responses by preventing secretion of the pro-inflammatory cytokines interleukin (IL)-1β, IL-6, IL-18, and TNFα by macrophages (Tracey 2010). This mechanism depends on the α7 nicotinic ACh receptor (α7nAChR) that inhibits the nuclear translocation of NF-κB and suppresses cytokine release by both

macrophages and monocytes. Overall, this parasympathetic vagus activation initiates an anti-inflammatory reflex-like process and has been shown to alleviate inflammatory disease, including endotoxemia and septic peritonitis (Tracey 2010).

At the environmental and therapeutic levels, ACh signaling determines individual reactions to widely employed anticholinesterase therapeutics (Darvesh et al. 2003) but also to agricultural pesticides (Howard et al. 2010) and to the prophylactic treatment with anticholinesterase agents in anticipation of exposure to poisonous nerve gases (Kaufer et al. 1998). High amounts of AChE negate the cholinergic anti-inflammatory signal of ACh (Ofek et al. 2007; Shaked et al. 2009), whereas AChE inhibition has been shown to restrict inflammation both in the periphery and in the CNS (Pollak et al. 2005). For example, recombinant AChE injection increases immune response, and miRNA-132 that blocks AChE production potentiate the anti-inflammatory reflex (Shaked et al. 2009; Waiskopf et al. 2014a), also shown in intestinal tissues from patients with inflammatory bowel disease (Maharshak et al. 2013).

Up- or downregulation of individual ChEs may reflect changes in ACh neurotransmission and/or homeostasis as will be shown below. However, the different ChE concentrations and catalytic efficiencies as well as potentially complex compensation mechanisms (e.g., parallel elevation in one ChE and reduction in the other) called for coining a term reflecting the outcome status in terms of global cholinergic signaling potency. This term, "cholinergic status (CS)", expresses the total capacity in a tested sample to hydrolyze ACh, and it depends on the cumulative contribution of both AChE and BChE toward ACh hydrolysis (Fig. 1b). It has been suggested that the CS reflects significant, albeit relatively small, changes in the levels of each ChE, while excluding cases where compensation avoided modification in the global cholinergic state.

The nonclassical roles of ChEs such as the capacity to hydrolyze additional molecules, their interactions with other proteins, and their presence in sites where ACh is absent suggested that they also have additional functions in health and disease. A relevant example would be the diabetes-related interaction of BChE with amylin that attenuates both amylin fibril and oligomer formation, protecting pancreatic β cells from amylin cytotoxicity (Shenhar-Tsarfaty et al. 2011b). The massive involvement of ChEs in diverse pathways further implicates disruption or overreaction of the complex cholinergic signaling process in numerous acquired diseases. A relevant case is that of Alzheimer's disease, where cholinergic neurons are lost early during disease progression for yet unknown reasons, leading to a hypocholinergic state (Berson et al. 2012) that is the reason for the currently available palliative treatment with cholinesterase inhibitors. Intriguingly, the amyloid precipitation in the Alzheimer's brain is inversely regulated by the different AChE splice variants. Thus, the major AChE-S splice variant promotes amyloid fibrillation, exacerbating the brain neuropathology hallmark of Alzheimer's disease (Inestrosa et al. 1996). In contrast, the stress-induced soluble AChE-R variant and the C-terminally mutated BChE-K variant are both associate with reduced amyloid fibrillation (Diamant et al. 2006; Berson et al. 2008). Moreover, BChE-K limits tau phosphorylation in demented patients (Ballard et al. 2005), which may reflect its impaired protein-protein interactions.

MiRNA Regulation of Cholinesterases

A key level of regulation on cholinergic signaling involves microRNA (miRNA) suppression of ChE mRNAs. MiRNAs are rapidly acting short RNA regulators of most genes. They provide posttranscriptional control over ACh signaling by interacting with, arresting the translation of, and rapidly inducing destruction of mRNA transcripts produced from all of the cholinergic genes and their regulators (Soreq and Seidman 2001), ChEs included. Therefore, changes in the levels of ChE-targeting miRNAs may modulate ChE levels, thus modifying ACh signaling. We designate miRNAs targeting cholinergic genes and their regulators "CholinomiRs." Similar to other miRNAs, they have many different targets, often involved in the same biological pathway, and indeed CholinomiRs frequently target more than one cholinergic gene (Nadorp and Soreq 2014). However, the power of CholinomiRs to suppress each one of their targets is context dependent and is modified based on a combinatorial state involving the cell type, the differentiation stage, and the evolutionary and inherited nature of the affected pathway.

To assist the survival of species where miRNAs evolve, specific miRNA-target gene pairs may be co-subjected to evolutionary changes. Examples include miR-132 and AChE, where the miRNA-target interaction remained fully preserved through evolution (Shaked et al. 2009). Other ChE-targeted miRNAs, such as the primate-specific miR-608 that also targets AChE, evolved more recently, implying that human AChE is subjected to more complex miRNA regulation than the mouse one (Hanin et al. 2014). However, this carefully controlled balance is impaired in various disease states. In the brain of Alzheimer's disease patients, which most likely include a considerable fraction of aging cardiac patients, the levels of the AChE-targeted miR-132 decline more sharply than most other miRNAs (Lau et al. 2013). Therefore, the decline in brain AChE is more limited than predicted by the massive loss of cholinergic neurons in the demented brain, possibly explaining why anticholinesterase therapeutics exerts a palliative effect on treated patients. Nevertheless, such therapeutics may modulate the cardiac status as well, which should be taken into consideration. This is especially relevant when considering body-brain signaling through the vagus nerve and/or blood cell-mediated ACh hydrolysis, which leads to changes in CholinomiR levels in response to stressful stimuli (Shaltiel et al. 2013).

The miRNA controllers of AChE predictably limit inflammation (which is blocked by ACh and is hence potentiated by AChE). At the same time, miRNA suppression of AChE elevates ACh levels, promoting anxiety (which associates with stimulated ACh (Meshorer and Soreq 2006)). Parallel effects may occur in different tissues, and indeed miR-132 levels are increased in inflamed intestinal biopsies from patients with intestinal bowel disease; the correspondingly reduced AChE levels may lead to locally exacerbated cholinergic signaling, protecting the diseased tissue from excessive inflammation (Maharshak et al. 2013). That inflammatory bowel states may co-occur with cardiac malfunctioning (Talbot et al. 1986) calls for considering miRNA changes as well for diagnostic purposes. The combinatorial mode of functioning of CholinomiRs further implies changes in their efficacy when the tightness of binding of a specific target is modified. Examples for such situations include SNPs in

the non-coding domains of the cholinesterase genes. Such SNPs were regarded inert until recently, because they do not change the coding of the proteins encoded by the SNP-carrying transcripts; however, they may interfere with miRNA-target interactions and lead to a resultant domino effect by intensifying the change in other targets through the otherwise free miRNA chains. Such changes in miRNA functions can occur under stress and inflammatory insults as well as following cardiac events and modify ACh signaling, for example, by changing AChE levels.

Inherited interference with CholinomiR interactions that impairs their binding to one or more of their target genes may spread into changes in other targets, escalating the miRNA/target interaction effect. For example, the frequently occurring rs17228616 SNP, which impairs the primate-specific regulation of AChE by miR-608, yields a 60 % excess of brain AChE activity in carriers of the minor allele, which constitute 4.5 % of the Caucasian population but as much as 34.5 % of Africa-originated populations. The occurrence of this SNP relieves miR-608 to hyper-block other targets such as the Rho-GTPase CDC42 or the cytokine IL-6. In young healthy adult carriers of the minor rs17228616 allele, both of these changes (AChE elevation and suppression of CDC42, IL6, and many other miR-608 targets) lead to elevated anxiety, inflammation, exacerbated systolic and diastolic blood pressure, and reduced cortisol levels (Hanin et al. 2014). Correspondingly, the close-by rs2820037 SNP at chromosome 1q43 has been identified as a regulator of hypertension. Taken together, these findings suggest a causal association between cholinergic signaling and stress-induced hypertension.

ChEs as Biomarkers: Detection Methods

The importance of the ChE enzymes as crucial players in various biological systems and the changes in their levels in many pathological processes did not remain unnoticed. Diverse high-sensitivity methods have been developed to detect and quantify ChE protein and catalytic activity levels. These methods can be divided into physical detection methods (such as spectrophotometric, calorimetric, radiometric, or the use of biosensors) and should also be classified by the nature of the biological sample to be used (e.g., serum or tissue) or into fully or partially quantitative and qualitative methods. Specifically, a plethora of qualitative methods were developed to detect ChE activities, for example, test strips or sticks with graduated color scale (Ryhanen and Hanninen 1987). Those have been valuable for assessing the risk of poisoning by AChE-targeted insecticides and/or nerve gases, which further implied the need for convenient methods to be used in field conditions and could overcome the limitations of the common quantitative laboratory techniques that were time-consuming and expensive and required special laboratory instruments. Today, with the advancements in technology, the advantages of such qualitative methods to measure ChE activity are no longer distinct. Hence, here we focus on the major simple and fast quantitative techniques for the use of ChEs as biomarkers.

The main quantitative method to measure ChE activity is based on Ellman's assay, a method developed for detection of thiol groups (Ellman et al. 1961) (Fig. 2a). In this method, the hydrolysis of ChE substrate analogs with thioesters results in reaction between the thiolated product and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) as a pro-dye to produce a dye with an absorption peak at 412 nm. Measuring the change of absorption with time is proportional to enzyme activity. Such measurements were traditionally performed by hydrolysis of butyrylthiocholine (BTCh) for quantification of BChE activity as a biomarker for organophosphate poisoning. However, this method solely measures BChE activity, and although BChE is the major ChE in the circulation, it hydrolyzes ACh slower than AChE (Soreq and Seidman 2001). Therefore, the relatively sparse AChE may have an important impact on ACh hydrolysis in the circulation in spite of its low levels. This realization led to the suggestion that in this case the CS will be a better biomarker and, hence, the use of acetylthiocholine (ACTh), an analog of ACh that can be hydrolyzed by both enzymes, is more relevant. If the activity of specific ChEs is sought as well, preincubation of the test samples with specific ChE inhibitors can provide this information. For example, tetramonoisopropyl pyrophosphortetramide (iso-OMPA) and 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW284C51) are used to selectively inhibit BChE and AChE activities, respectively. This method is fast, simple, accurate, and of relatively low cost. However, it requires minimal initial absorption of the sample in the dye's absorption peak. For example, the Soret absorption of hemoglobin can increase the baseline absorption of the sample in the blue region that may interfere with the readings of ChE activity with DTNB as the pro-dye. Moreover, the spontaneous hydrolysis of the substrate and possible reduction of the pro-dye by other molecules in the sample can increase the observed kinetics and should also be taken into account. Some spectrometric methods try to address these disadvantages by using dyes with absorption peaks in longer wavelengths and/or by measurement methods that do not involve sensitive redox reactions. For example, ACh hydrolysis can be detected by the use of choline, an ACh hydrolysis product, as a substrate for choline oxidase (ChOx) coupled with peroxidase, which together with phenol and amino-antipyrine provides absorption change around 500 nm (Abernethy et al. 1986).

Fluorometric assays were also developed; most of them use pro-fluorescence dye as ChE's substrates (e.g., indoxyl acetate, naphthyl acetate, resorufin acetate) or as molecules that interact with the hydrolysis product of thioesters (e.g., fluorescein-5-maleimide, methylcoumarin maleimide) (Parvari et al. 1983). These methods can provide higher sensitivity to lower ChE concentrations. However, whereas the absorption change can be easily translated to concentration using the Beer-Lambert law, fluorometric measurements face an additional limitation in that the measured emission can be affected by reabsorption, energy transfer, or quenching due to interaction or instability of the fluorescent dye.

Various electrochemical sensors of ChEs can also be found in the literature. Most of these sensors are based on ChE immobilized on electrodes and are aimed to detect and quantify ChE inhibitors. The sensors planned to detect ChE activities are generally based on two step reactions. The first, similar to the spectrometric assays,

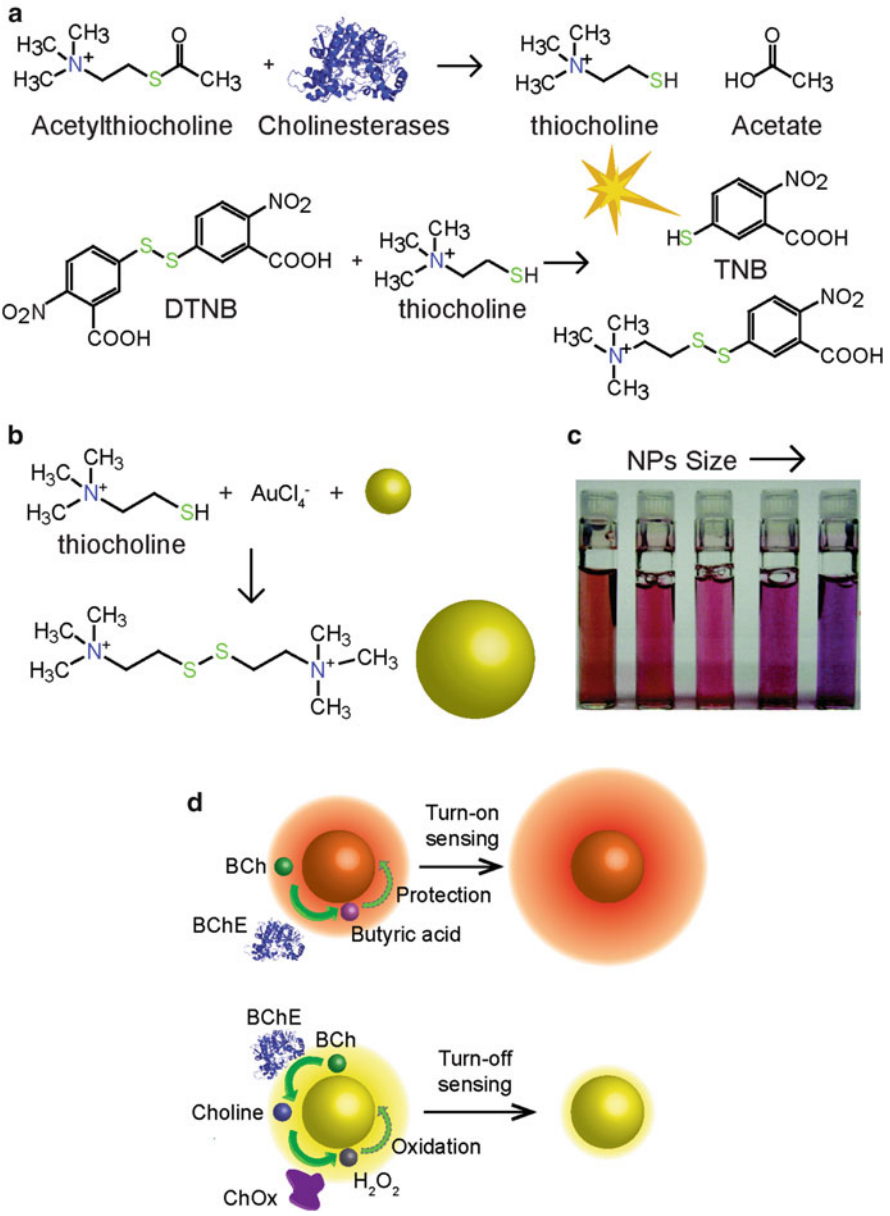

Fig. 2 Cholinesterase activity measurements. (**a**) Scheme of the standard Ellman's assay. ChE's substrate analogs are hydrolyzed to yield thiolate product that reduce DTNB to TNB, leading to the development of a distinct absorption spectrum. (**b**) The ChE's hydrolysis product, thiocholine, can be used for promoting the growth of gold NPs. (**c**) Solution absorption changes with gold NP size. (**d**) Turn-on and/or turn-off of semiconductor NP fluorescence used to measure ChE's activity. Figures (**b**) and (**c**) were reprinted and adapted with permission from Pavlov et al. (2005) and Murphy et al. (2008), copyrights (2005 and 2008, respectively) American Chemical Society. Figure (**d**) was reprinted and minimally adapted from Chen et al. (2013), Copyright (2013) with permission from Elsevier

is the hydrolysis of substrates, such as esters or thioesters, producing acids and choline or thiocholine, respectively. The second uses the hydrolysis product itself for the electrochemical measurement. For example, ChE activities can be monitored by using potentiometric sensors with pH-sensitive electrodes for detection of pH change. Such sensors detect pH decreases due to the release of acids (e.g., acetic acid) as the hydrolysis product. However, the main limitation of such systems is their sensitivity due to the high biological buffer capacity that minimizes pH changes. Another example is that of the voltammetric assays based on thiocholine oxidation in human plasma samples which showed similar detection limits to those of Ellman's assay (Pohanka 2014).

Exciting advancements in nanotechnology opened new opportunities for quantitative measurements of ChE activities. Developments in the synthesis and surface engineering of nanoparticles (NPs) for biological applications optimized their use for imaging, sensing, and delivery with controlled release. Colloidal NPs or NPs conjugated to biomolecules or electrodes were used to detect the ChEs and their inhibitors and to study their functions and interactions for research and for the use of recombinant ChEs for therapeutics (Waiskopf et al. 2011, 2014b).

NPs were mainly used to quantify ChE activities by tracking the changes in their optical properties upon ChE hydrolytic activity. For example, thiocholine was used as a reducing agent for the growth of gold NPs in the presence of $AuCl_4^-$ as a precursor (Fig. 2b). The subsequent change in the NP plasmonic absorption peak due to the changes in NP dimensions (Fig. 2c) was used to quantify ChE activity and detect ChE inhibitors. In a different method, thiocholine was used to accelerate NP aggregation, which resulted in detectable spectral changes due to inter-particle plasmonic interaction (Wang et al. 2009; Pavlov et al. 2005). Fluorometric assays were also suggested using semiconductor NPs, thereby utilizing their superior photochemical stability in comparison to traditional fluorescent dyes. For example, ChE activity was detected by multistep reactions in which the ACh hydrolysis product, choline, was used as a substrate of ChOx to produce peroxide that quenches the semiconductor NP fluorescence (Chen et al. 2013; Gill et al. 2008) (Fig. 2d).

These methods and others can be used to quantify ChE activities, each with its own advantages and limitations. However, in all of them as with any kinetic measurement, the conditions of the measurement, such as pH, temperature, and the solution's ionic strength, should be strictly controlled to provide accurate and comparable results.

Potential Applications to Disease or Condition Prognosis?

Well-adjusted cholinergic signaling depends on the concerted expression of multiple receptors, enzymes, and transporters, and imbalanced response can lead to disease (Ofek and Soreq 2013). For example, myasthenia gravis, Sjogren's syndrome, asthma, and inflammatory bowel disease are all characterized by uncontrolled cholinergic response, and possible treatments are available to restore the cholinergic balance (Ofek and Soreq 2013). Much effort is hence devoted to develop reliable methods for manipulating cholinergic signaling and to develop biomarkers to follow

treatment efficacy and distinguish between health and disease (Shenhar-Tsarfaty et al. 2014). Several studies show ChEs involvement in CHD. Serum BChE was implicated in the development of CAD (Alcantara et al. 2002), and Calderon-Margalit et al. demonstrated that individuals in the lowest quintile of BChE activity had significantly higher rates of all-cause and cardiovascular mortality (Calderon-Margalit et al. 2006). Moreover, Goliasch et al. demonstrated a strong association between decreased ChE activity and long-term adverse outcome in patients with known CAD, which was stronger in stable CAD patients than in those with acute coronary syndrome (Goliasch et al. 2012) (Fig. 3a).

The common denominator for the abovementioned studies was the focus on BChE activity for evaluating ACh-hydrolyzing capacity in the serum and the use of BTCh, a butyrylcholine analog as a substrate. This might reflect the routine availability of automated equipment for performing such measurements in medical centers, avoiding the need for specialized biochemistry laboratory services. However, the use of ATCh as a substrate that can be hydrolyzed by both enzymes is physiologically more meaningful and advantageous as it can better reflect the full parasympathetic potency in inactivating ACh and might offer insights as to the nature of its contribution to cardiovascular disease. In a recent study, we evaluated the individual significance of two biomarkers of the parasympathetic system, AChE activity and the CS, in randomly selected patients undergoing coronary angiography by employing ATCh as a substrate and using selective inhibitors to differentiate between AChE and BChE activities and testing their association with major adverse cardiac events (MACE). We found that patients with MACE presented lower CS and AChE values at catheterization than no-MACE patients whose levels were comparable to those of matched healthy controls (Fig. 3a). Also, patients with AChE or total CS values below median showed conspicuously elevated risk for repeated MACE compared to those above median. This parasympathetic dysfunction in patients undergoing coronary angiography predicted up to 40 months MACE. Therefore, monitoring AChE and CS parameters might help in the risk stratification of patients with cardiovascular disease. In another cohort of 403 apparently healthy working volunteers, we found that increased ChE activities, measured in the peripheral blood, correlated with basal heart rate, attenuated heart rate increase during exercise, and delayed heart rate recovery following exercise testing (Fig. 3a, Table 1), suggesting that ChE activities can serve as readily measurable serum markers for autonomic cardiovascular imbalance even in healthy adults (Canaani et al. 2010).

While CAD is the most common form of heart disease, the same mechanism and/or risk factors can reflect the risk of unfavorable recovery from brain ischemia or stroke. Stroke continues to present a significant public health challenge, being not only the second leading cause of death but also a leading cause of adult disability (Roger et al. 2012). Bacterial pneumonia remains the main medical complication after stroke, accounting for almost 20 % of in-hospital deaths and poor outcomes at discharge (Koennecke et al. 2011). There is growing evidence that acute injury to the central nervous system, including stroke, directly impairs the antibacterial host defense (Meisel and Meisel 2011). Occlusion of the middle cerebral artery (MCAO) in mice, a model of human stroke, showed association to the development

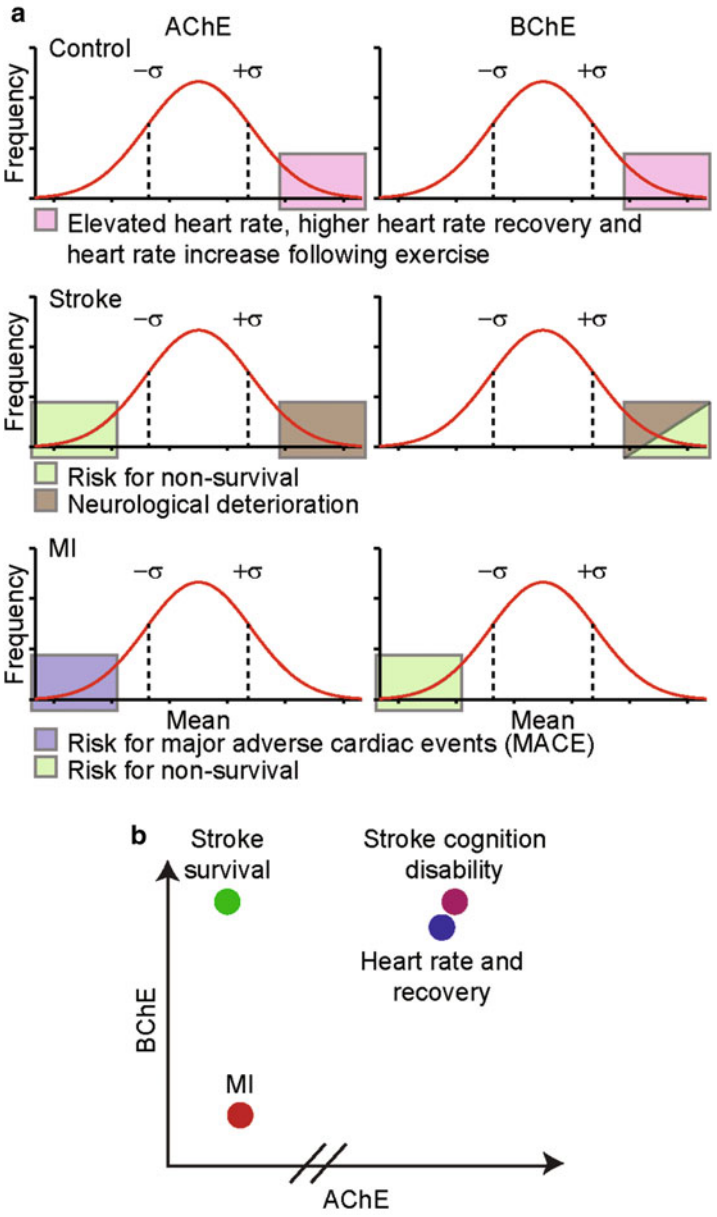

Fig. 3 Serum AChE and BChE activities as biomarkers for cardiovascular diseases. (**a**) Histograms of population frequencies of AChE and BChE activities of control (*top*), stroke (*middle*), and myocardial infarction patients (*bottom*). *Colored squares* represent extreme values of enzymatic activities in patients with risk for poor outcome. For example, apparently healthy individuals with elevated AChE or BChE are at increased risk of higher heart rate and slower heart rate recovery following exercise. (**b**) Enzymatic activities of AChE and BChE in patients compared to controls.

of spontaneous bacterial infections within 24 h after the onset of stroke, leading to high mortality (Prass et al. 2003). Infections are preceded by rapid suppression of peripheral cellular immune responses. This appears to be triggered by a stroke-induced over-activation of the sympathetic nervous system (Prass et al. 2003).

The vagus nerve stimulates celiac ganglion adrenergic neurons that innervate the spleen, leading to release of ACh and activation of the nAChR on splenic macrophages. This blocks production of the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α). Recently, Rosas-Ballina et al. identified a subpopulation of CD4+ T cells that secrete ACh, express β-adrenergic receptors, and are located adjacent to adrenergic nerve endings in the spleen. Transplanting these T cells into mutant mice devoid of T cells and exposed to endotoxemia-inducing insults rescued the attenuation of TNF-α by vagus nerve stimulation (Rosas-Ballina et al. 2011). Furthermore, reduced pre-transplantation expression of the ACh biosynthesis regulator choline acetyltransferase (ChAT), by small interfering RNA in these T cells blocked rescue of TNF-attenuation after vagus nerve stimulation. Thus, ACh secretion by these T cells is required for this inflammatory reflex.

Measuring AChE and BChE activities provides effective biomarkers for assessing the immunosuppressive power of the autonomous nervous system under stroke (Fig. 3a). In our own study, we demonstrated that in patients after acute ischemic stroke, declined serum AChE activity predicts the neurological outcome, survival, and inflammatory reactions (Ben Assayag et al. 2010) (Fig. 3b, Table 1). Moreover, in an experimental model of stroke, occlusion of the middle cerebral artery in mice deficient in invariant natural killer T cells (achieved by ablating *CD1d*) leads to increased bacterial burden in the lungs, greater pulmonary inflammatory damage, and decreased survival as compared with wild-type mice, while the stroke severity was similar in both strains. Prophylactic antibiotic treatment completely prevented stroke-associated death in *CD1d*−/− mice, suggesting that their lack of invariant natural killer T cells rendered them more susceptible to death from infections after stroke (Wong et al. 2011). Additionally, Sykora et al. reported decreases in the autonomous system's measure of baroreflex sensitivity (BRS) as an independent predictor for poststroke infections (Sykora et al. 2011), and both the BRS and serum AChE activity correlated with multiple inflammatory biomarkers (Shenhar-Tsarfaty et al. 2011a). Together, these different yet interrelated approaches to estimate the cholinergic suppression of inflammation (Fig. 3b) demonstrate their value for assessing the consequent risk of infections in cerebrovascular diseases.

Fig. 3 (continued) Patients undergoing cardiac catheterization presenting low AChE and BChE activities are at risk to develop MACE complications, whereas all stroke patients present lower AChE activities compared to controls; those with extremely reduced AChE levels are at risk for non-survival, whereas those with smaller reductions of AChE activities are at risk for poor neurological recovery. Last, apparently healthy individuals with higher enzymatic activities of both enzymes are at increased risk for higher heart rate

Table 1 Cholinesterase involvement in cardiovascular-related diseases

Disease	Risk for	Biomarker		SNPs and mutations	References
		AChE	BChE		
Asymptomatic individuals	Elevated heart rate	↑	↑	rs17228616	Hanin et al. (2014), Canaani et al. (2010), and Sklan et al. (2004)
Asymptomatic individuals (men only)	Slower (higher) heart rate recovery and heart rate increase following exercise	↑	↑	?	Canaani et al. (2010)
Stroke	Survival	↓	↑	rs1803274	Ben Assayag et al. (2010) and Shenhar-Tsarfaty et al. (2010)
	Neurological decline	↑	↑	?	Ben Assayag et al. (2010)
Myocardial infarction	Mortality	–	↓	?	Goliasch et al. (2012)
	Major adverse cardiac events (MACE)	↓	–	?	Arbel et al. (2014)

Summary and Future Prospects

AChE and BChE, the hydrolyzing enzymes of the neurotransmitter ACh, serve as key regulators of neurotransmission, inflammation, and anxiety in the central and peripheral nervous systems. Since their discovery, several methods were developed for enabling sensitive, low-cost, and easily performed detection of the ChEs and their activities. Using those methods on large cohorts of apparently healthy as well as stroke and myocardial infarction patients revealed the possible use of these biomarkers for parasympathetic/sympathetic balance and prognostic purposes.

High AChE or BChE activities are correlated with inflammation, heart rate, and slower heart rate recovery, whereas patients with low AChE activities are at risk for non-survival following stroke or major adverse cardiac events following myocardial infarction. Current and future studies are aimed to gain better understanding of the regulation and involvement of the ChEs in cardiovascular diseases.

Summary Points

- The cholinesterases are key factors in diverse biological pathways and systems.
- Cholinesterase activities can be measured by numerous simple low-cost methods.

- Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) can serve as diagnostic markers for the parasympathetic/sympathetic balance and predict cardiovascular adverse events.
- Patients with low AChE activities are at risk for non-survival following ischemic stroke or major adverse cardiac events following myocardial infarction.
  Patients with high AChE or BChE activities are at increased risk for inflammation, anxiety, and slower heart rate recovery.

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Triglycerides (TG) to High-Density Lipoprotein (HDL-c) Ratio (TG/HDL-c Ratio) as a Marker of Cardiometabolic Risk

10

Tommaso de Giorgis and Angelika Mohn

Contents

Abstract

Cardiovascular diseases represent the main cause of mortality and morbidity worldwide. Several metabolic conditions, as obesity, diabetes, metabolic syndrome, hypertension, and hypercholesterolemia, seem to play a pivotal role in the pathogenesis of cardiovascular diseases.

During the last 20 years, different surrogate markers have been proposed as possible tools not only to identify and to evaluate the progression of cardiovascular disease but also to recognize precocious stages of different cardio-metabolic diseases in general population.

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_21

One of the most promising biomarker is the triglyceride-to-high-density lipoprotein cholesterol (TG/HDL-c) ratio that has been proposed as a new emerging marker able both to reflect the cardio-metabolic status and to predict the increased risk of developing metabolic and cardiovascular complications in adults as well as in children. In fact, several evidences demonstrated the TG/HDL-c ratio is well related not only with current cardio-metabolic diseases, but it seems to be able to predict the risk to develop cardiovascular accidents.

The goal of this book chapter is to describe the potential role of TG/HDL-c ratio as a marker to evaluate and to predict cardiovascular and metabolic diseases in adults and in children.

Keywords

TG/HDL-c ratio • Lipid profile • Cardiovascular risk • Cardio-metabolic diseases • Triglycerides • HDL cholesterol

Abbreviations

AD	Atherogenic dyslipidemia
CHD	Cardiovascular heart diseases
cIMT	Carotid intima-media thickness
CVD	Cardiovascular diseases
HDL-c	High-density lipoprotein cholesterol
HOMA-IR	Homeostasis model assessment
IR	Insulin resistance
LDL-c	Low-density lipoprotein cholesterol
TC	Total cholesterol
TG	Triglycerides
TG/HDL-c	Triglyceride-to-high-density lipoprotein cholesterol
WBISI	Whole-body insulin sensitivity index

Key Facts of the Triglycerides (TG) to High-Density Lipoprotein (HDL-c) Ratio (TG/HDL-c ratio) as a Marker of Cardio-Metabolic Risk

- TG/HDL-C ratio represents a good surrogate marker to define the cardiovascular risk related to the atherogenic dyslipidemia.
- Several evidences have clearly demonstrated that TG/HDL-C ratio represents a key metabolic marker of metabolic and cardiovascular complications in obese subjects.
- TG/HDL-C ratio is directly related to IR status both in adult and children.
- In adult subjects, TG/HDL-C ratio is associated with the severity of coronary arterial stenosis.
- In obese children and adolescent, TG/HDL-C ratio is related to early signs of atherosclerosis as cIMT, left ventricular hypertrophy, arterial stiffness, and brachial distensibility.

Definitions

Arterial stiffness Is an age-related process that occurs when the elastic fibers within the arterial wall (elastin) begin to fray due to mechanical stress. Increased arterial stiffness is associated with an increased risk of cardiovascular events.

Arteriosclerosis Is the thickening, hardening, and loss of elasticity of the walls of arteries. It should not be confused with atherosclerosis, which is a specific form of arteriosclerosis caused by the buildup of fatty plaques and cholesterol in the artery.

Atherosclerosis Is a specific form of arteriosclerosis in which an artery wall thickens as a result of invasion and accumulation of white blood cells, remnants of dead cells, cholesterol, and triglycerides. Atherosclerosis is therefore a syndrome affecting arterial blood vessels due to a chronic inflammatory response of white blood cells in the walls of arteries. This is promoted by low-density lipoproteins (LDL-c, plasma proteins that carry cholesterol and triglycerides) without adequate removal of fats and cholesterol from the macrophages by functional high-density lipoproteins (HDL-c). It is commonly referred to as a "hardening" or furring of the arteries. It is caused by the formation of multiple athermanous plaques within the arteries.

Atherogenic dyslipidemia Is a risk-conferring lipid/lipoprotein profile characterized by a higher proportion of small LDL particles, reduced HDL-C levels, and increased values of triglycerides.

Carotid intima-media (cIMT) Also called intimal-medial thickness, is a measurement of the thickness of tunica intima and tunica media, the innermost two layers of the wall of an artery. The measurement is usually made by external ultrasound and occasionally by internal, invasive ultrasound catheters; see intravascular ultrasound. Measurements of the wall thickness of blood vessels can also be done using other imaging modalities.

Dyslipidemia Is an abnormal elevation of plasma total cholesterol, triglycerides (TGs), or low high-density lipoprotein level that contributes to the development of atherosclerosis. Causes may be primary (genetic) or secondary.

Introduction

Cardiovascular diseases (CVD) represent the main cause of mortality and morbidity worldwide. Several metabolic conditions such as obesity, diabetes, metabolic syndrome, hypertension, and hypercholesterolemia seem to play a pivotal role in the pathogenesis of cardiovascular accidents (Go et al. 2013). Despite considerable improvements in medical care over the past 25 years, CVD remain one of the major public health challenges. In fact, the World Health Organization estimates

that more than six million of deaths are due to cardiovascular diseases every year worldwide, and this number seems to rise to more than 20 million during the next decades (Lozano et al. 2012). In addition, a recent National Vital Statistical Report calculates that CVD are in the top of the list of the 15 leading causes of death in the USA, with an annually total cost of 108.9 billion of dollars each year for health-care services, medications, and lost productivity (Murphy et al. 2013). In the same way, in Europe, CVD are responsible for nearly 50 % of all deaths, and they are the main cause of all disease burdens, with management costs estimated at 192 billion euro annually (The World Health Organization 2012). Most importantly, several studies underline that this burden is projected to escalate dramatically not only in USA and in Europe but also in underdevelopment countries (Mahmood et al. 2014).

More alarming are the data regarding the increase of cardiovascular disease in pediatric population. In fact, recently considering the important rise of obesity and different obesity-related complications, the spectrum of cardiovascular diseases is becoming more relevant already in children and adolescents (Cote et al. 2013). Many studies, as the Bogalusa Heart Study and Framingham Study, have convincingly shown that childhood obesity is not only an important risk factor for obesity during adult age, but it also increases the risk to develop precociously CVD, metabolic syndrome, and atherosclerosis (Li et al. 2012; Mahmood et al. 2014).

In order to contain this important growth of cardiovascular accidents in adult population, during the last 20 years different surrogate markers have been proposed as tools to evaluate the progression of CVD but also to recognize in general population precocious stages of different cardio-metabolic diseases, when they are still silent. However, up to now, there are a series of uncertainties on the possibility to obtain a single biomarker that could identify and predict early signs of cardio-metabolic diseases (Cohn 2004).

Recently, one of the most used biomarkers is the triglyceride-to-high-density lipoprotein cholesterol (TG/HDL-c) ratio, which has been proposed as a new emerging marker able both to reflect the cardio-metabolic status and to predict the increased risk of developing metabolic and cardiovascular complications in adults as well as in children (Arca et al. 2012; Salazar et al. 2012).

The goal of this book chapter is to describe the potential role of TG/HDL-c ratio as a marker to evaluate and to predict cardiovascular and metabolic diseases in adults and in children.

The Role of Atherogenic Dyslipidemia and Cardiovascular Diseases

In 1959, the Framingham Heart Study identified cholesterol levels as one of the most important risk factors for the development of cardiovascular (Dawber et al. 1959). Confirming these results, during the last decades, several prospective studies have clearly demonstrated that high levels of low-density lipoprotein cholesterol (LDL-C) and low levels of high-density lipoprotein cholesterol (HDL-c) represented the most important lipid abnormalities involved in the pathogenesis of atherosclerosis. In fact,

these prospective observations described this lipid profile as a predictor factor for the risk to develop CVD (Castelli 1996; Rosenson 2005).

With the increasing prevalence of obesity and its related complications, there is a new interest to define the role of other lipid molecules involved in the pathogenesis of atherosclerosis (Fig. 1). In 1990, for the first time, Austin et al. described "atherogenic dyslipidemia" (AD) or "atherogenic lipoprotein phenotype," as a major lipid abnormalities implicated in the pathogenesis of obesity-related complications. These authors proposed a risk-conferring lipid/lipoprotein profile characterized by a higher proportion of small LDL particles, reduced HDL-C levels, and increased values of triglycerides (Austin et al. 1990). This spectrum of lipid abnormalities is normally evaluable in patients with obesity and with obesity-related complications, as metabolic syndrome, insulin resistance, and type 2 diabetes mellitus (Kannel et al. 2008; Wu and Parhofer 2014; Gasevic et al. 2014). In particular, diabetic dyslipidemia is a widespread condition in which insulin resistance seems to be the driving force for the genesis of the characteristic lipid abnormalities (Kannel et al. 2008). After the definition proposed by Austin, different studies underlined that this spectrum of lipid abnormalities could represent an important factor for CVD risk also in general populations. In fact, in these studies considering the diabetic dyslipidemia as a risk factor for CVD, it was more highly associated with incident CVD events (hazard ratio of 1.22 per 1 standard deviation) than single value of LDL-c (hazard ratio of 1.10 per 1 standard deviation) (Musunuru 2010). In addition, in a separate post hoc analysis of two large and different clinical trials conducted in subjects with stroke while receiving a statin and otherwise best medical therapy, those having atherogenic dyslipidemia had a higher residual cardiovascular risk than those without AD (Sirimarco et al. 2014). Therefore, it is easily understandable that these lipid abnormalities seem to be strictly associated with cardiovascular risk. In

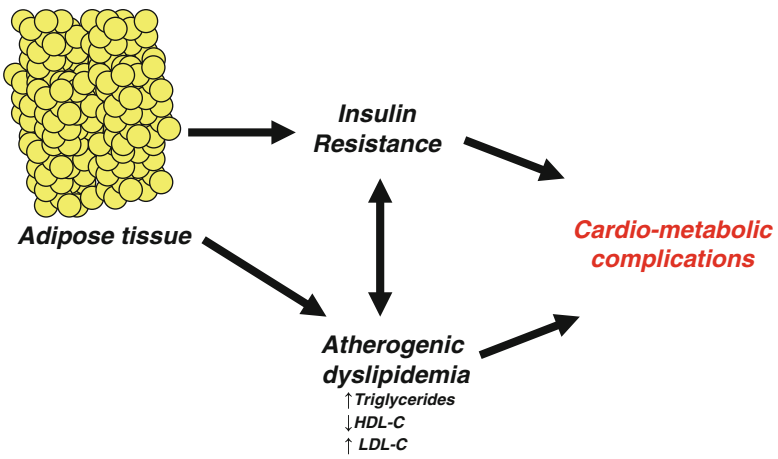

Fig. 1 Cardiovascular complications related to obesity and obesity-related metabolic complications

Table 1 LDL-C and atherosclerosis

Small dense LDL particles in athero/vasculo activities
Easily trapped in arterial wall
Easily oxidized
Pro-inflammatory activity
Pro-thrombotic activity

Table 2 Triglycerides and atherosclerosis

Triglycerides in athero/vasculo activities
Constitute core of the triglyceride-rich lipoproteins (remnant cholesterol)
Easily trapped in arterial wall
Increase binding and lipolysis at the artery wall
Pro-inflammatory activity
Pro-thrombotic activity
Impaired vasodilatory activity

fact, it is well known that small dense, lipid-poor LDL particles have greater susceptibility to oxidation, and these molecules seem to be able to penetrate in the arterial wall earlier than LDL-C, generating the inflammatory processes in vascular endothelium (Preiss and Sattar 2009) (Table 1).

Although triglycerides (TG) are not directly circulating, they represent the core of the triglyceride-rich lipoproteins (remnant cholesterol). Remnant cholesterol molecules are simply trapped in arterial wall for their size attaching to extracellular proteoglycans. In this context, lipoprotein-lipase activity induces the release of free fatty acids, monoacylglycerols, and other components of triglycerides that induced a local injury, activating the production of inflammatory factors and promoting the thrombin generation (Chapman 2010; Chapman et al. 2011; Nordestgaard and Varbo 2014) (Table 2). The other component of atherogenic dyslipidemia is HDL-c. Normally HDL-c is considered an athero-protective factor. In fact, several evidences underline a very important relevant role of HDL-c in the athero/vasculo-protection process. These molecules seem able to play an important anti-inflammatory and antioxidant activity stimulating different mechanisms that control endothelial repair system or endothelial vasodilator activity. In addition, it is well known that HDL-c controls the cellular cholesterol efflux and cholesterol homeostasis. In fact, it is able to acquire additional lipids and apolipoproteins derived from the hydrolysis of triglyceride-rich lipoproteins and reduce the peripherally presence of triglycerides. Subsequently, these "new and mature" HDL-c molecules are metabolized or directly by liver uptake or by steroidogenic tissues via different and specific tissue receptors/enzyme. This complex and intriguing efflux process seems to be able to partly account for the strong inverse relation between TG and HDL-c (Rader and Hoving 2014) (Table 3).

Although, all these studies have underlined the role of the single components of atherogenic dyslipidemia and the correlation with several cardiovascular diseases, new evidences reported that sometimes the single components of the atherogenic dyslipidemia cannot reflect their overall cardiovascular risk, whereas their

Table 3 LDL-c and atherosclerosis

HDL in athero/vasculo-protection activities
Control cellular cholesterol efflux
Anti-inflammatory activity
Antioxidative activity
Endothelial repair
Vasodilatory activity
Anti-thrombotic activity

combination in a single ratio seems to have a better predictive power for both cardiovascular and metabolic diseases (Salazar et al. 2012).

Potential Applications to Prognosis: TG/HDL-c Ratio as a New Marker for Cardio-Metabolic Diseases in Adult Subjects

Several evidences demonstrated that the traditional cholesterol measurements tend to be most accurate in predicting cardiovascular risk only for those at the lower and higher ends of the risk spectrum, while they seems to be not so strongly related to the cardiovascular risk for those patients that are in the middle part of lipid profile (Chapman et al. 2011; Preiss and Sattar 2009). Therefore, there has been a growing focus of research on the possibility to identify a surrogate marker of lipid abnormalities that could be able to define the cardiovascular risk in the general population. Recent data demonstrated that the ratio between different components of lipid profile represents one of the most specific predictors of cardiovascular risk (Preiss and Sattar 2009). At the beginning of this century, several groups showed that LDL-c/HDL-c or total cholesterol (TC)/HDL-c could be considered as good markers of cardiovascular disease. In fact, changes in this ratio have been shown to be a better indicator of a successful CHD risk reduction compared to changes in absolute levels of lipids or lipoproteins. In particular, many studies conducted in population with different cardiovascular risk have clearly reported that LDL-c/HDL-c ratio is significantly more robust predictor of CVD than the individual levels of LDL-c or HDL-c (Kannel et al. 2008; Manninen et al. 1992). However, both LDL-c/HDL-c and TC/HDL-c ratio seems to be well related only with CVD, while it is poorly linked to metabolic diseases implicated in the pathogenesis of CVD (Wu and Parhofer 2014).

Therefore, recently there has been a growing interest on the possibility to identify a new ratio between different component of lipid profile that is better related to cardiovascular and metabolic diseases and that could represent also a good predictor for the future risk to develop cardio-metabolic diseases. One of the most promising factors that seems to have the previously noted characteristics is the ratio between TG and HDL-c. In fact, it is well known that TG, low-density, and HDL-c are mainly deregulated in different metabolic diseases (as type 1 diabetes, insulin resistance, type 2 diabetes, etc..), and they seem directly related to risk of cardiovascular diseases (Wu and Parhofer 2014). However, several studies underlined that the combination of TG and HDL-c in a single ratio confers a good power not only to

define the current cardio-metabolic status but also to predict the future cardiovascular risk (Kannel et al. 2008; Sirimarco et al. 2014; Gasevic et al. 2014; Salazar et al. 2012).

For the first time, in 2008, Kannel et al. confirmed the role of the TG/HDL-c ratio as a positive predictor of cardiovascular and metabolic risk in the large cohort of adult obese subjects included in the Framingham offspring study. In this paper, the authors analyzed the relationship between TG/HDL-c ratio not only with insulin resistance (IR) but also with the risk to develop cardiovascular events longitudinally. Therefore, considering a large study population of 3,014 patients (mean age 54 years; 55 % women), the authors demonstrate that in the spectrum of the several considered lipid markers, TG/HDL-c ratio represented the best parameters correlate with IR. In addition, the authors showed that IR prevalence increased across the tertiles of lipid ratios ($p < 0.0001$); also the area under curves for predicting IR on the base of TG/HDL-c ratio confirmed a strong correlation between IR and the ratio in this large population. In order to evaluate the power of the ratio to predict possible cardiovascular events, the authors continued to monitor the enrolled population. In particular, during a follow-up period of mean 6.4 years, a group of 112 patients experienced a first CHD event. In this longitudinal arm of the study, the authors demonstrated that even after adjustment for lipid variables (including TG/HDL-c ratio), IR was significantly and strongly associated with CHD risk. Interestingly, these prospective analyses suggested that TG/HDL-c ratio is a good surrogate index of IR (multivariable-adjusted hazards ratio 2.71, 95 % confidence interval 1.79–4.11). In conclusion, these observations recommend a role of TG/HDL-c ratio as a surrogate marker for IR. In addition, this parameter seems to be a good predictor of potential cardiovascular risk related to insulin resistance. This study represents a milestone to use the TG/HDL-c ratio as a marker of cardio-metabolic disease (Kannel et al. 2008).

Moreover, also a new recent study confirms that obese subjects with a high TG/HDL-c values have a considerably increased risk of CHD and CVD. In this study, the authors considered a population of 54,061 patients from the Swedish National Diabetes Register, and they showed that obese and prominently obese subjects with TG/HDL-c $\geq$1.9 had an hazard ratios around 1.7 for fatal/nonfatal CHD and 1.6 for CVD ($p < 0.001$), while obese and prominently obese patients with TG/HDL-c ratio $<$1.9 presented hazard ratios of 1.2 for CHD and 1.3 for CVD ($p < 0.005$). However, it is important to remark that in all these studies, the authors demonstrated the relation between the lipid ratio and insulin resistance; nevertheless, they did not prove a direct relationship between TG/HDL-c ratio and direct signs of CVD (Eeg-Olofsson et al. 2014).

In order to evaluate the direct influence of TG/HDL-c ratio on CVD, Yang et al. designed a study to explore the relationship between different lipids ratio and the degree of coronary artery stenosis, defined according to Gensini score. For this study, the authors enrolled 207 patients divided in four groups according to the severity of coronary stenosis: group 1 or control group (34 patients), group 2 with a score less than 30 score (84 patients), group 3 with a score from 31 to 90 score (66 patients), and group 4 scored greater that 90 (23 patients). These authors

demonstrated that the coronary lesions increased moving across tertiles of TG/HDL-c, but also with the increase of other lipid parameters taking into account, as LDL-c/HDL-c, levels of TC, LDL-c, triglycerides, TC/HDL-c, and reduction of HDL-c. In particular, the authors showed that patients with a higher coronary artery stenosis (groups 2, 3, and 4) presented significantly increased values of ratio compared to group 1 ($p < 0.05$); however, when they compared the values of ratio across the groups 2, 3, and 4, no differences in terms of TG/HDL-c were found. In addition, also the Pearson correlation analysis revealed that only LDL-c/HDL-c ($r = 0.54, p < 0.05$) and TC/HDL-c ($r = 0.50$, $p < 0.05$) were significantly and positively correlated with the coronary artery lesions. The results suggested that the severity of coronary artery lesions were correlated with abnormal lipid metabolism; however, the predictive value of TG/HDL-c was not confirmed in this study (Yang et al. 2011).

At the same time, a different group investigated the association between lipid levels, specifically TG/HDL-c, and a direct sign of cardiovascular disease, as the extent of coronary disease. In this study, the authors enrolled a group of 374 high-risk patients (220 males and 154 females, age 57.2 ± 11.1 years) admitted to their attention to perform coronary angiography. In all patients, lipid parameters were measured, and they were scored according to the coronary disease extent using the Friesinger index. The main results of this study show that the severity of coronary disease (dichotomized by a Friesinger index of 5) is directly related to triglycerides [odds ratio of 2.02 (1.31–3.1; $p = 0.0018$)], HDL-c [odds ratio of 2.21 (1.42-3.43; $p = 0.0005$)], and TG/HDL-c [odds ratio of 2.01(1.30–3.09; $p = 0.0018$)]. After categorizing subjects according to quartiles of the Friesinger score, the authors demonstrated that the frequency and the severity of coronary disease increased progressively moving from the lower to the upper tertiles of the ratio (47.9 vs. 63 vs. 66 vs. 75.3; $p = 0.0018$). In addition, the odds ratio for the extent of coronary disease between the lower and the upper quartiles and TG/HDL-c was 3.31, (95 %CI 1.78–6.14, $p = 0.0002$), suggesting that across the TG/ HDL-c quartiles, the increase of the ratio led to a 30 % increase in disease extent. In addition, in order to investigate the potential independent contribution of the TG/HDL-c ratio on severity of atherosclerotic lesions, a multivariate analysis by logistic regression was performed, and these analysis revealed that the TG/HDL-c ratio showed a strongest association with extent of coronary disease (0.779 ± 0.074, $p = 0.0001$). Finally a ROC curve was calculated to individuate a value of the TG/HDL-c ratio able to identify subjects with Friesinger score in the upper quartile of the ratio. An AUC-ROC value of 0.63 for TG/HDL-c ($p = 0.0001$) can identify subjects with high risk for cardiovascular events. It is important to show that although this study demonstrated for the first time a direct relationship between the ratio and direct signs of cardiovascular disease, it present same points that should be addressed. In particular, it might be noted that the authors did not include in their study population a control group; therefore, the results of this study could be apply only in subjects with high cardiovascular risk (da Luz et al. 2008).

Considering all these studies demonstrating the power of TG/HDL-c ratio as a useful biomarker able to reflect the cardio-metabolic status and to predict subjects at increased risk of developing cardiovascular complications, a recent guideline for the

clinical approach to obese patients recommends that the TG/HDL-c ratio should be used to define the impaired metabolic status and chronic inflammation in these subjects. This guideline is intended as a useful guide that can be used by health-care professionals in everyday clinical practice in order to easily detect obese subjects with increased cardio-metabolic risk. The authors of this guideline proposed that a value of the TG/HDL-c ratio major than 2 seems to reflect the current metabolic status, and it is able to predict the future risk of cardiovascular diseases (Lau et al. 2007).

Potential Applications to Prognosis: TG/HDL-C Ratio as a New Marker for Cardio-Metabolic Diseases Already in Pediatric Population

According to these previously findings, recently some evidences have proposed that even in the pediatric population, the TG/HDL-c ratio is related to IR, chronic inflammation, and cardiovascular risk (Quijada et al. 2008; Giannini et al. 2011; Musso et al. 2011). With regard to the pediatric population, Giannini et al. proposed for the first time in obese children and adolescents that the TG/HDL-c ratio could represent a good marker of IR also in this population. In this study, the authors enrolled a group of 1,452 obese and multi-ethnic children and adolescents, and they evaluated lipid profile and insulin sensitivity. In particular, it is important to note that in this study, Giannini et al. measured insulin sensitivity not only using surrogate indices of insulin sensitivity, as whole-body insulin sensitivity index (WBISI) and homeostasis model assessment (HOMA)-IR, but in a subgroup of 146 obese youths, they also defined insulin sensitivity by the hyperinsulinemic-euglycemic clamp. As main results, the authors showed that across rising tertiles of TG/HDL-c ratio, WBISI progressively decreased, whereas 2-h glucose and the AUC-glucose progressively increased. In addition, this group using a receiver operating characteristic (ROC) curve analysis proposed a threshold of TG/HDL-c ratio able to identify subjects in the upper quartile of WBISI. The estimated cutoff for TG/HDL-c ratio was 2.27, and the odds of presenting with IR, in youths with TG/HDL-c ratio higher than the cutoff, was 6.023 (95 % CI 2.798–12.964; $p = 0.001$) in white girls and boys, whereas for both Hispanics and African Americans, the AUC-ROCs were not significant. Therefore, this study showed in a large multi-ethnic cohort that the TG/HDL-c ratio is associated with IR mainly already in pediatric population and thus may be used as risk factor to identify subjects at increased risk of IR (Giannini et al. 2011).

Subsequently an Italian group demonstrated that this lipid ratio is not only directly related with IR status, but it also represents a good marker to evaluate possible preclinical signs of cardiovascular diseases in obese children and adolescent. The authors evaluated in a large population of normal-weight and obese children and adolescents (884 subjects) a possible correlation between TG/HDL-c ratio and early signs of cardiac remodeling, such as left ventricular hypertrophy. In line with previously reported results in adult subjects, Di Bonito et al. demonstrated a correlation between increasing values of ratio and well-known cardio-metabolic

parameters, as insulin resistance, liver enzymes, or other indexes of metabolic impairment status, already during childhood. In addition, for the first time, this study reported not only that left ventricular hypertrophy increased across tertiles of the TG/HDL-c ratio in children and adolescents but also that pediatric subjects with a TG/HDL-c ratio major than 2.0 had a two to threefold higher risk of concentric LV hypertrophy compared to those with a TG/HDL-c ratio lower than 2.0 (Di Bonito et al. 2012).

In addition a new recent study reported the role of this lipid ratio in the pathogenesis of vascular remodeling evaluated by arterial stiffness and brachial distensibility in obese youth. In this population, the authors described a progressive rise in arterial stiffness across TG/HDL-c ratio. In addition, the ratio seemed to be an independent determinant of brachial distensibility in CV risk factor. These results confirmed that a high TG/HDL-c ratio is related not only with specific cardiometabolic profile but also with preclinical signs of cardiac abnormalities already in pediatric population. Moreover, also these data confirmed that a value of TG/HDL-c ratio major than 2.0 could be considered a useful clinical marker to detect children with high cardio-metabolic risk (Urbina et al. 2013).

In a recent study, our group (de Giorgis et al. 2013) tried to extend this association between the TG/HDL-c ratio and early signs of cardiovascular disease in children, assessing the relationship between the ratio and carotid intima-media thickness (cIMT) that is a more feasible, direct, and noninvasive method, detecting preclinical signs of arterial wall dysfunction in obese pediatric population. In our study, obese children showed significantly higher values of the TG/HDL-c ratio (1.9 ± 1.1 vs. 1.2 ± 0.6, $p = 0.002$) compared with controls. In addition, after dividing the population in tertiles of the TG/HDL-c ratio (<1.04, 1.04–1.67,>1.67), insulin resistance and marker of chronic inflammation progressively increased moving from the lower to the upper tertile (HOMA-IR $p = 0.0001$, WBISI $p = 0.0003$ and sRAGE $p = 0.05$). Interestingly, also cIMT progressively increased moving across tertiles ($p = 0.0003$) (Fig. 2). Additionally, a multiple linear regression analysis revealed a significant and positive correlation between the TG/HDL-c ratio and cIMT ($r = 0.493$, $P = 0.0005$). Considering this very interestingly relation between cIMT and the lipid ratio, a ROC curve analysis was calculated in order to estimate a threshold of TG/HDL-c ratio that was able to identify the subjects in the upper quartile of cIMT. A cutoff point for TG/HDL-c ratio of 1.12 had 81 % sensitivity and 49 % specificity in the identification of children with cIMT values in the upper quartile (de Giorgis et al. 2013). It needs to be acknowledged that in our study population, values of the TG/HDL-c ratio were lower compared to values proposed in previous studies (Giannini et al. 2011; Musso et al. 2011). However, this study population included only prepubertal and Caucasian children. Therefore, these aspects could explain the differences in terms of threshold of TG/HDL-c ratio, where also adolescents and a mixture of ethnic groups were studied. These data could also reflect the well-known influences of puberty and ethnicity on insulin sensitivity and cardio-metabolic parameters. In conclusion, in this study we showed that the TG/HDL-c ratio is an additional independent factor associated with cIMT; therefore, these data provided a further line of evidence for a role of the TG/HDL-c ratio in the cardiovascular risk.

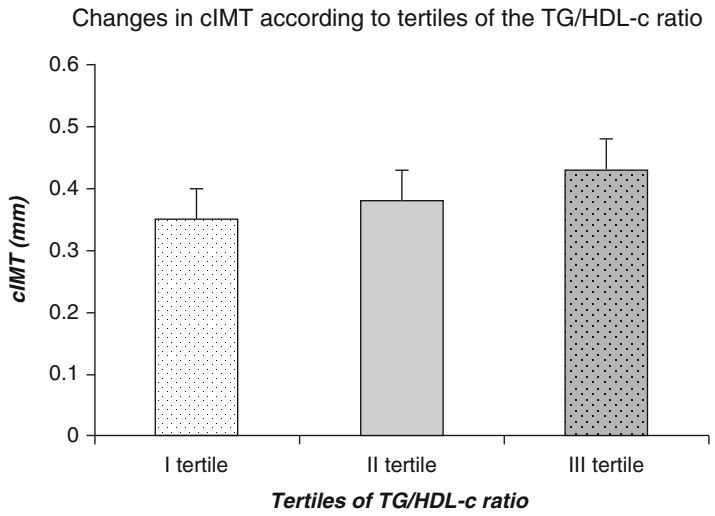

Fig. 2 Changes in cIMT according to tertiles of the TG/HDL-c ratio

Taking together, all these findings support the role of the TG/HDL-c ratio as a useful marker able to define the cardio-metabolic status also in obese children and adolescents. Therefore, all these evidences underline the role of the ratio as a new emerging marker of cardiovascular disease in adult population as well as also in childhood.

What Are the Limits of the TG/HDL-C Ratio as a Marker of Cardio-Metabolic Risk?

As previously showed, strong evidences support the role of the TG/HDL-c ratio as a reliable marker of cardiovascular disease in adults as well as also in children. However, a series of limits have been reported regarding the possibility to introduce this ratio as a single recommend marker for screening general population at increased risk for developing metabolic and cardiovascular complications.

The first limit is related to the possibility to identify a standardized cutoff point for the TG/HDL-c ratio above which subjects present an increased cardio-metabolic risk. Up to now, although several studies have been conducted, with the main aim to use the ratio as a marker of the cardio-metabolic status, there are a series of differences in proposed threshold (Di Bonito et al. 2012; Giannini et al. 2011; Salazar et al. 2012). Probably, these differences in terms of proposed threshold for TG/HDL-c could be related by the differences of characteristics in the populations included in these studies. In fact, it is well know that there are substantial differences in lipid profile and cardio-metabolic risk in adult population according to different ethnicity. In particular, these discordances seem to be more evident in pediatric population. In fact, there are a series of strong data indicating that in children more that in adult subjects, lipid profile is influenced by different parameters as age, gender, pubertal stage, and ethnicity.

Table 4 Advantages and disadvantages of TG/HDL-c ratio

Advantages	Disadvantages
Not expensive	Not specific for a single disease
Easy to calculate	A single cutoff is not available
Correlated with direct and surrogate signs of cardio-metabolic diseases	Influenced by changes of different components of lipid profile according to age, gender, pubertal stage and infections
Correlated with insulin resistance	

Some studies demonstrated that the relationship between the ratio and cardio-metabolic parameters is not confirmed when the studies included in their study population both obese African-American and non-Caucasian subjects. Therefore, these differences in the TG/HDL-c ratio according to different ethnic group could be related to the differences in genetic patterns that are able to influence the specific ethnic cardio-metabolic risk (Davis 2008). In addition, it needs to be acknowledged that the large difference for the proposed threshold of the TG/HDL-c ratio in pediatric group could be related to the pubertal characteristics of children included in the study population. In fact, the major part of these studies included in their study population both pubertal and prepubertal children, and only few of these studies performed a sub-analysis in order to define a specific value of ratio according to the pubertal stage of the population. Consequently, the variability in the proposed cutoff point of the TG/HDL-c across the different studies could be related to the well-known physiological changes in lipid profile, insulin resistance, and other cardio-metabolic parameters related to puberty (Radtke et al. 2012). It easy understandable that the possibility to apply of ratio as a markers of cardiovascular diseases in general pediatric population is strictly related to the chance to have percentiles of TG to HDL ratio for age, gender, pubertal stage, and ethnicity. Therefore, considering this limit, new studies should be performed in order to obtain a specific cutoff point.

The last limitation associated to the use of the TG/HDL-c ratio in the clinical practice is related to the absence of long-term follow-up studies evaluating the power of this marker during life. In fact, although we have sufficient data on the role of TG/HDL-c ratio as a marker able to measure the current cardio-metabolic status in adults as well in children (Di Bonito et al. 2012; Giannini et al. 2011; Salazar et al. 2012), there are no data regarding a possible role of the ratio as a factor able to predict the future cardio-metabolic risk. Therefore, longitudinal studies are needed in order to verify whether TG/HDL-C ratio could be the best marker able to reflect and predict the cardio-metabolic status during long life (Table 4).

Conclusion

In conclusion, the TG/HDL-C ratio seems to represent a new and useful marker related to cardio-metabolic risk factors and early signs of vascular damage both in adults and in children (Giannini et al. 2011; Di Bonito et al. 2012; de Giorgis et al. 2013;

Eeg-Olofsson et al. 2014; Kannel et al. 2008). These data suggest that the use of TG/HDL-c may be helpful in identifying patient at high risk for cardiovascular diseases requiring aggressive intervention to prevent atherosclerotic CV diseases.

Although different studies confirm the important role of this marker in adult patients and in children with different metabolic and cardiovascular diseases, there are a series of limits that should be considered in particular when the ratio would be used in general population (Giannini et al. 2011; Di Bonito 2011; de Giorgis et al. 2013; Eeg-Olofsson et al. 2014; Kannel et al. 2008).

Therefore, other longitudinal and large studies are needed in order to validate also in adults as well as in pediatric population the power of the TG/HDL-C ratio as a marker able to reflect not only the current cardio-metabolic status but also the risk to develop cardio-metabolic disease later in life.

Summary Points

- To contain the important increase of cardiovascular accidents in general population, during the last 20 years, different surrogate markers have been proposed as tools not only to evaluate the progression of diseases but especially to recognize early in general population a precocious stage of diseases, probably when they are still silent.
- Several studies have clearly demonstrated that atherogenic dyslipidemia, characterized by decreased levels of HDL-c associated with increased TG and normal or minimally elevated levels of LDL-c, seems to be directly implicated in the pathogenesis of atherosclerosis in obese subjects.
- Recently, one of the most promising biomarker is the triglyceride-to-high-density lipoprotein cholesterol (TG/HDL-c) ratio that has been proposed as a new emerging marker able both to reflect the cardio-metabolic status and to predict subjects at increased risk of developing metabolic and cardiovascular complications in adults.
- Considering the strong correlation between TG/HDL-c ratio and different surrogate markers of cardio-metabolic diseases in obese subjects, a recent guideline for the clinical approach to obese patients recommends that the TG/HDL-c ratio should be used to define the impaired metabolic status and chronic inflammation in these subjects.
- There has been growing interest on the role of the TG/HDL-c ratio as a new emerging marker able to reflect the cardio-metabolic status and to predict subjects at increased risk of developing metabolic and cardiovascular complications in pediatric population.
- Several evidences demonstrated that TG/HDL-c ratio represents a strong surrogate marker of insulin resistance and of early signs of cardiovascular diseases; therefore, it could be used as an important risk factor to develop cardiovascular diseases already in obese pediatric population.
- A series of limits should be consider regarding the possibility to introduce this ratio as a recommend marker for screening general population at increased risk of developing metabolic and cardiovascular complications.

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Biomarkers of Myocardial Cell Damage: Heart-Type Fatty Acid Binding Protein (H-FABP) for the Early Evaluation of Suspected Acute Coronary Syndrome

11

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_43

Abstract

Suspected acute coronary syndrome (ACS) represents a substantial healthcare problem and is responsible for a large proportion of emergency department admissions. Better triaging of patients with suspected ACS is needed to facilitate early initiation of appropriate therapy in patients with acute myocardial infarction and to exclude low-risk patients who can safely be sent home thereby limiting healthcare costs. Heart-type fatty acid-binding protein (H-FABP) is established to be the earliest available plasma marker for myocardial injury. In this chapter, the clinical utility of H-FABP for suspected ACS is evaluated. H-FABP shows added value in addition to cardiac troponin, especially in the early hours after onset of symptoms. Moreover, H-FABP identifies patients at increased risk for future cardiac events. It is concluded that measuring H-FABP along with troponin shortly after onset of symptoms improves risk stratification of patients suspected of having ACS in a cost-effective manner.

Keywords

Fatty acid-binding protein • H-FABP • FABP3 • Acute coronary syndrome • Acute myocardial infarction • Plasma biomarker • Early diagnosis • Point-of-care test

Abbreviations

ACS	Acute coronary syndrome
AUC	Area under curve
CABG	Coronary artery bypass grafting
CI	Confidence interval
CK	Creatine kinase
CK-MB	Creatine kinase MB
cTn	Cardiac-specific troponin
cTnI	Cardiac-specific troponin I
cTnT	Cardiac-specific troponin T
CV	Coefficient of variance
ECG	Electrocardiogram
GP	General practitioner
h	Hour
H-FABP	Heart-type fatty acid-binding protein
hs-cTn	High-sensitive cardiac-specific troponin
hs-cTnI	High-sensitive cardiac-specific troponin I
hs-cTnT	High-sensitive cardiac-specific troponin T

MI	Myocardial infarction
NPV	Negative predictive value
NT-proBNP	N-terminal B-type natriuretic peptide
PoC	Point of care
PPV	Positive predictive value
ROC	Receiver operating characteristic
UAP	Unstable angina pectoris

Key Facts of H-FABP

- Several molecules play a role in the function of heart cells and are detectable exclusively inside the heart cells.
- In a healthy situation, these molecules are not detectable on a significant level in the blood. In cases of damage to heart tissue, usually in cases of heart infarction, several of these substances can be released into the peripheral blood.
- Therefore, they become detectable in peripheral blood, for example, obtained through venous blood sampling or a capillary finger prick.
- This way, a rise in these molecules as measured in a blood sample is highly indicative for cell damage and possibly heart infarction in a patient, especially when complaints fitting this diagnosis are present simultaneously.
- Such molecular markers of a certain disease are named *biomarkers*.
- Before clinical use in patients is possible, the value of these biomarkers must become undisputed in studies including a large number of real patients with and without the studied disease (e.g., heart infarction).
- Heart-type fatty acid-binding protein (H-FABP) is an example of such a biomarker for heart cell damage that is currently studied for its use in daily medical practice.

Definitions

Acute coronary syndrome Clinical description of complaints suspicious for a cardiac ischemic cause. Those are new or worsened complaints when compared to an earlier, stable phase. To distinguish between acute coronary syndrome and other cardiac or noncardiac causes, plasma troponin measurement is necessary unless ST elevations on ECG are seen. In case of a confirmed acute coronary syndrome, unstable angina or myocardial infarction is present.

Angina pectoris Chest pain due to diminished blood flow in one or more coronary arteries. Stable angina pectoris: "predictable" chest pain occurring at a certain degree of exercise for a long time, due to myocardial ischemia caused by stable stenosis in a coronary artery without local thrombotic activity. Collateral circulation often compensates for diminished perfusion through the affected coronary artery. Stable angina is usually treated with medication, aiming at reduction of complaints as well as

secondary prevention of cardiovascular disease. Invasive intervention is sometimes indicated when severe complaints or increased mortality risks are present. Unstable angina pectoris: chest pain of new onset or chest pain occurring at lower intensities of exercise than in the recent past, mostly due to acute plaque rupture in a coronary artery, activating local thrombotic activity. Unstable angina pectoris is a clinical diagnosis; myocardial ischemia is present, but plasma troponin levels remain normal. Condition is usually treated with invasive intervention that may be necessary on a short term.

Biomarkers of myocardial cell damage Any molecule that is released into the circulation following myocardial cell damage and becomes measurable in peripheral blood; troponin and heart-type fatty acid-binding protein are examples of such biomarkers.

Chest pain Pain in (ventral and/or lateral and/or dorsal) thoracic region of any cause. Among possible causes are gastroesophageal causes, thoracic wall pain, and angina pectoris.

Diagnostic accuracy Term used for the overall capacity of a test to play a role in the diagnostic process. Accuracy in this context is a collective term for specificity, sensitivity, and positive and negative predictive value.

Myocardial infarction (Near-)complete occlusion of a coronary artery, most often caused by plaque rupture and local thrombotic activity in consequence. Myocardial damage is present; troponin is released from the damaged myocardial cells. ST elevations on ECG can be present or absent. Usually treated with urgent percutaneous coronary intervention or bypass surgery. Prognosis: depends on magnitude of myocardial cell loss.

Point-of-care test Test device able to measure one or more laboratory parameters and to deliver a result within a limited period of time. Result is known within the time of consultation of the patient; transport of patient material to externally located laboratory facilities is unnecessary. In a broader perspective, any test that delivers immediate results can be regarded as point-of-care test, for example, devices to measure temperature of blood pressure.

Primary care, general practice, and family medicine Synonyms used to describe the field of medicine where no limitation on type of complaints and disease is maintained. No thresholds for care are established. Attention to a broad patient perspective (somatic, psychological, social context) is a main goal in primary goal.

Reliability Other than diagnostic accuracy, this term describes the test ability to measure accurately and in a reproductive manner, without regarding the diagnostic potency when used in a clinical perspective.

Introduction: Heart-Type Fatty Acid-Binding Protein (H-FABP) and Acute Coronary Syndrome

In an earlier publication, heart-type fatty acid-binding protein (H-FABP) has been extensively reviewed (Glatz and Renneberg 2014). Referral is made to this chapter for a basic, mainly biochemical approach with elaborate references. In this present chapter, H-FABP is reviewed in a rather clinical perspective, to underline the future perspectives of H-FABP in dealing with acute coronary syndrome (ACS) in primary and secondary care.

A Major Healthcare Problem: Suspected Acute Coronary Syndrome

Cardiovascular diseases remain the leading cause of death in industrialized countries with coronary artery disease being the most prevalent manifestation (Fact sheet no. 317 Geneva, 2013). The clinical presentations of this include stable angina pectoris, unstable angina pectoris (UAP), myocardial infarction (MI), heart failure, manifestations of silent ischemia, and sudden death. Marked improvements in clinical treatment during the previous decades have resulted in increased survival of patients with acute coronary syndrome (ACS, i.e., MI or UAP). In contrast, diagnostic means have remained poor, especially in an early stage of acute coronary artery disease when patients may need immediate clinical treatment. While chest pain is the main symptom of chronic coronary artery disease and ACS, early assessment is hampered by the large number of patients presenting with chest pain of another, less severe, cause.

Dilemma in Chest Pain: ACS or Alternative Cause?

ACS represents a life-threatening manifestation of atherosclerosis causing a sudden and critical reduction in coronary blood flow due to intraluminal thrombosis. The therapeutic objective is to achieve rapid, complete, and sustained reperfusion by primary angioplasty or fibrinolytic therapy. Therefore, rapid triaging of patients presenting with chest pain is needed to facilitate early initiation of appropriate treatment in patients with acute MI. At the same time, low-risk patients who can safely be sent home without further expensive diagnostic analysis should be identified as well. Because the latter group currently represents up to 80 % of patients with suspected ACS (Bruins Slot et al. 2011; Goodacre et al. 2013; McConaghy and Oza 2013), adequate ruling out of MI and of UAP is important in view of not only patient burden but also the large costs involved, which may include ambulance transfer, extensive diagnostic procedures, and hospital stay.

Thus, one of the main demands in the diagnostic process of coronary artery disease is to distinguish chest pain caused by coronary obstruction from chest pain

with a benign course (thoracic wall pain, gastroesophageal reflux disease, etc.) (Hamm et al. 2011). In the former situation, urgent specialist care is needed, which is rarely the case in the latter situation. Therefore, in a patient presenting with chest pain to primary or secondary care facilities, the presence or absence of ACS should be crystal clear as soon as possible in order to design the most cost-effective diagnostic strategy in patients presenting with chest pain suggestive of MI. Among the most promising tools to reach this goal are biomarker tests that deliver immediate results at the point of care.

High-Sensitive Troponin and Additional Biomarkers

As a sensitive biomarker of cardiac injury, high-sensitive troponin (hs-cTn) is of great importance in the current diagnostic strategy in suspected ACS. Using hs-cTn, acute MI can be ruled out based on a negative test result in an emergency setting as early as 3 h after onset of symptoms (Bandstein et al. 2014). However, extension of these diagnostic rule-out strategies could lead to further cost reduction and more convenience for patients. Besides, time of onset of complaints can be uncertain. Altogether, in this field of increasing rule-out capacity, additional biomarkers that appear in plasma at an earlier point in time after MI can be of interest. In the following paragraphs, the specific demands of ruling out ACS in primary care and at an early moment in secondary care are made explicit. Furthermore, it is described how available evidence indicates that heart-type fatty acid-binding protein (H-FABP, also designated FABP3) fulfills the criteria to be useful for triaging patients with acute chest pain particularly in the early hours after onset of symptoms. In this chapter, therefore, the clinical utility of H-FABP for the early evaluation of suspected ACS is depicted. Other biomarkers representing different aspects of an evolving acute MI such as markers for vascular stress (copeptin, N-terminal B-type natriuretic peptide or NT-proBNP), oxidative stress (myeloperoxidase), or plaque instability (placental growth factor) are not discussed in this chapter, since their diagnostic value is not well defined or considered not useful (Keller et al. 2011; Collinson et al. 2013).

ACS in Primary Care

Because a large number of patients with symptoms suggestive of MI will first be presented to primary care physicians, often during out-of-office hours, the general practitioner (GP) plays a crucial role in the early diagnosis and referral of these patients (Bruins Slot et al. 2011; Goodacre et al. 2013; McConaghy and Oza 2013). In case of suspected ACS, patients will be urgently referred to a secondary care facility, since early treatment of ACS markedly increases survival and quality of life (Reimer and Jennings 1979; Gersh et al. 2005). The majority of patients presenting with chest pain to a physician (either in primary or in secondary care), however, do not suffer from an acute cardiac condition at all. In specialized care facilities such as

coronary care units, only 50 % of patients presenting with chest pain are diagnosed with ACS, whereas in primary care, ACS is diagnosed in no more than 1.5–22 % of cases (Bruins Slot et al. 2011; McConaghy and Oza 2013; Willemsen et al. 2015). In the remainder of cases, chest pain is mostly caused by a condition with beneficial outcome (e.g., gastroesophageal reflux disease, thoracic wall pain, etc.). Given the high prevalence of noncardiac chest pain, expenses to exclude severe disease in such patients result in a significant societal burden (Mourad et al. 2013). Moreover, even after expensive diagnostic research, reassuring patients is challenging (Dumville et al. 2007). Referring every patient with chest complaints would overwhelm secondary care facilities; however the GP is faced with serious diagnostic dilemmas since milder diseases with beneficial outcome can mimic ACS and vice versa (Body et al. 2010). To distinguish chest pain caused by ACS from chest pain of another cause therefore remains challenging.

ACS in Primary Care: Diagnostic Means

Contemporary diagnostic means are insufficient to overcome the difficulties in distinction between chest pain due to ACS and thoracic complaints due to alternative causes. This is caused by several reasons with two common factors: all tools are of limited availability in general practice or lack acceptable negative predictive value (NPV) and sensitivity. A few points, partly based on current literature and partly based on experience in daily practice, can be made. First, literature confirms that symptoms and signs vary widely in chest pain possibly due to ACS, from none (in circa 25 %) to severe, and thus have limited diagnostic value in a significant amount of cases (Brieger et al. 2004; Bruyninckx et al. 2008; Body et al. 2010). Second, validated decision rules for general practice to rule out AMI or ACS have been developed, but evidence for superiority of using these decision rules above the GP's judgment without these rules is lacking (Bösner et al. 2010; Haasenritter et al. 2012). Third, the value of electrocardiography is limited, since only about 50–65 % of patients with ischemic cardiac disease have classic electrocardiogram (ECG) findings in the first time period after start of the complaints, while an ECG is sometimes not even available in general practice (Rutten et al. 2000). Fourth, since the definition of AMI is for an important part based on biomarker levels and AMI can in a significant amount of cases not be ruled out otherwise, blood analysis, especially measurement of the concentration of troponin, is a cornerstone in diagnosing as well as ruling out AMI. Venous blood samples, obtained in general practice, however cannot be analyzed on the spot, and adequate monitoring of the patient in expectation of the results is impossible. Moreover, serial measurement of plasma hs-cTn, the cornerstone in diagnosing as well as ruling out MI (Newby et al. 2012; Thygesen et al. 2012), is impossible to perform in primary care. This impairment could partly be overcome by usage of point-of-care (PoC) tests. Unfortunately, contemporary PoC troponin tests are less accurate due to detection limits

higher than the widely used cutoff values for a positive test, usually set at the 99th percentile of a healthy population (Nilsson et al. 2013).

Ruling Out ACS in Primary Care: Specific Demands

Since diagnostic means accessible for GPs lack potency to safely rule out ACS, a low threshold for referring patients with possible ACS is maintained. Although ACS is present in the minority of cases, a majority is referred to a cardiologist to rule out ACS. In a Dutch cohort of such patients, 27 % of patients were not referred, in 8 % of whom ACS was diagnosed in a later stage, leading to a false-negativity rate of 2 %. Seventy-three percent of patients were referred, 75 % of whom were not diagnosed with ACS (false-positivity rate or "unnecessary referral rate" 54.8 %) (Bruins Slot et al. 2011, 2013b). Patients that were referred and appeared to be ACS negative were diagnosed with alternative diseases with advantageous courses. Thus, over-referral of patients presenting with chest pain in primary care leads to a low number of missed cases of ACS but is an (expensive) burden to secondary care facilities (Graff et al. 1997). Since unnecessary referral considerably outnumbers missed cases in the triaging of patients with suspected ACS in primary care, focus is rather on ruling out ACS and other urgent medical conditions as early as possible. Thus improvement of diagnostic tools aims at making referral unnecessary, without missing more cases of ACS, enabling limitation of overall healthcare costs. Besides, anxiety in patients undergoing unnecessary diagnostic procedures is reduced.

A PoC test with a high negative predictive value for ACS that delivers a clear result within several minutes is needed to reach this goal. Notably, such a PoC test is among the most demanded future tests by GPs (Cals et al. 2014). An improved triage of patients with signs and symptoms suggestive of ACS would reduce unnecessary referral and associated cost and anxiety. Therefore, to enrich the diagnostic tools of a GP in thoracic symptoms and to reduce unnecessary referral in cases of clinical doubt, novel, immediately measurable biomarkers with strong potency to rule out myocardial infarction in single measurements are needed. Combined with signs and symptoms, such tools should be able to safely rule out ACS in a significant number of otherwise referred patients, without a rise in missed cases of ACS. Importantly, this would lead to a significant cost reduction. The number of referred patients would decrease, and in the remaining patients who are referred, ACS could be confirmed or ruled out as is common in secondary care.

In the field of pulmonary embolism and respiratory tract infections, diagnostic tools combining clinical signs and symptoms with the result of a PoC test have recently been introduced. Both increased efficiency by reducing unnecessary referral (in cases of suspected pulmonary embolism) or unnecessary treatment (in respiratory tract infections) (Cals et al. 2011; Geersing et al. 2012; Little et al. 2013). For ACS, a similar procedure has not yet been defined.

Table 1 Characteristics of plasma biomarkers for acute myocardial infarction

Marker protein	Molecular mass (kD)	First elevation in plasma after AMI[a] (h)	Peak plasma concentration (h)	Normalization of plasma level[b] (days)
H-FABP	14.5	1–2	6–12	1–1.5
Myoglobin	17.8	2–3	6–12	1–2
Cardiac troponin I	22.5	3–8	12–24	7–10
Cardiac troponin T	37.0	3–8	12–24	7–10
Creatine kinase MB	86	2–6	12–24	2–3

Several characteristics (molecular mass, first elevation in plasma after acute myocardial infarction (AMI), peak plasma concentration, and normalization of plasma level) of several widespread used biomarkers of cardiac ischemia

Abbreviations: *AMI* acute myocardial infarction, *h* hours, *H-FABP* heart-type fatty acid-binding protein, *kD* kilodalton

[a]First elevation above the upper reference level of the marker protein

[b]Dependent on (time of) reperfusion of the occluded vessels

ACS in Secondary Care

Various marker proteins are known to be released into plasma after MI, each showing a distinct tissue specificity and unique release pattern (see Table 1). Of these, the cardiac troponins, i.e., troponin T (cTnT) and troponin I (cTnI), are more specific and, as measured by the latest generation of troponin tests, more sensitive than the traditional cardiac enzymes, such as creatine kinase (CK) and its isoenzyme creatine kinase MB (CK-MB), and therefore have become the standard in establishing a diagnosis and stratifying risk (Thygesen et al. 2012). In patients with MI, plasma troponins initially rise about 3–4 h after symptom onset and remain elevated for up to 2 weeks due to slow proteolysis of the contractile apparatus in damaged cardiac myocytes. There is no fundamental difference between cTnT and cTnI. The NPV of contemporary fifth-generation hs-cTn tests in an emergency care department has increased to 98–99 % (Mueller 2014). Thus, evidence is growing that cardiac ischemia can be ruled out within 3 h after onset (Bandstein et al. 2014). Moreover, UAP seems to be diagnosed less because of the increasing sensitivity of hs-cTn (Mueller 2014). In new onset or altered chest pain where hs-cTn is negative, (severe) stable coronary artery disease is becoming increasingly diagnosed instead of UAP. Conversely, in cases where hs-cTn is slightly positive, MI is diagnosed according to the third universal definition of MI (Thygesen et al. 2012). An elevated hs-cTn value, i.e., above the 99th percentile of a normal reference population, is a strong indicator of myocardial cellular damage and has a very low false positivity.

Ischemia is not the sole cause of myocardial injury, however. Several other diseases also lead to myocardial cellular damage, including pneumonia, pericarditis,

and left ventricular stress. Using older-generation troponin assays, 30 % of patients testing positive had no coronary occlusion (Reichlin et al. 2009). With the current high-sensitivity assays, this percentage is probably even higher. To solve this issue, in the third universal definition of MI, AMI is diagnosed when, besides an elevated plasma hs-cTn, a change over time is measured. When such a change is detected, a coronary cause of the cardiac injury is likely (Thygesen et al. 2012). The magnitude of the change that is indicative of acute coronary occlusion is still open to debate. In the lower range of troponin results, an absolute change of 7 ng/L between two measurements is probably indicative of acute coronary disease, whereas in the higher range, a relative change of 20 % is needed (Biener et al. 2013).

Ruling Out ACS in Secondary Care: Specific Demands

Further improvement of the care process could be realized if patients presenting with chest pain of acute onset in secondary care – either after referral by a GP or otherwise – would undergo rule-out as soon as possible. Ideally such rule-out would occur within 1 h after onset of complaints. Furthermore, rule-out would ideally be based on a solitary measurement, while positive results due to other causes of myocardial injury should be limited as far as technically possible.

Ruling Out ACS in Primary and Secondary Care

As expounded above, main demands for ruling out ACS in primary as well as secondary care are early rule-out using a highly sensitive test with a high NPV for ACS. Such test could be an algorithm combining signs, symptoms, and a biomarker result. Moreover, especially in primary care, results should be available for assessment within several minutes. However, time to assessment is of significance in secondary care too, when definitive rule-out is demanded as early as possible (within 1 h). Key words in the field of future diagnostic means in ACS therefore are *point-of-care devices* and *high negative predictive value*. In the next subheadings, both requirements for efficient triaging are depicted.

Point-of-Care Tests

Contrary to pharmaceuticals, the legislation for diagnostic tests in general, and point-of-care tests in particular, is very limited. PoC tests may enter the European market after receiving no more than a CE certificate, which includes several fundamental, mostly technical, and laboratory aspects of the test. Proven reliability and diagnostic accuracy in daily clinical care do not belong to CE certification requirements. Consequently, primary care professionals took the initiative to start listing criteria to which PoC tests in their view should apply, and they simultaneously performed

studies in daily clinical care, on the performance of relevant PoC tests (Howick et al. 2014; Schols et al. 2015).

In primary care, PoC tests can be divided over (1) tests for (home) monitoring of patients who are unable to visit their GP; (2) tests for screening purposes, mostly as a service toward (future) patients; and (3) tests for ruling out particular diseases in patient presenting with symptoms possibly representing an underlying serious disease, like ACS. Preferably, all tests belonging to one or more of the above indications are fast, meaning they produce a test result within the duration of one consultation (i.e., 10 min). Furthermore, a PoC test for primary care must be reliable in the hands of non-laboratory trained personnel, it must have a better diagnostic accuracy than existing alternatives, its diagnostic accuracy must be investigated with the new PoC test as part of existing and accepted diagnostic algorithms, test results must adequately steer treatment or referral decisions, the test should be cost effective, costs of PoC testing must be properly reimbursed, PoC tests must be appreciated by both medical professionals and patients, and, last but not the least, a PoC test must be easy to implement in both the daily work-up of a GP and existing (laboratory) facilities of a particular clinic. Very few of currently in primary care used PoC tests apply to all requirements (Cals et al. 2013).

Infectious diseases and ACS belong to the relatively small group of (potentially) life-threatening diseases presented in daily primary care, needing PoC tests, like H-FABP, for quickly reaching accurate diagnostic conclusions and referral decisions. But before deciding on a definite introduction of H-FABP in primary care, the above-listed criteria must be evaluated in daily general practice circumstances.

Early Diagnosis of ACS: Plasma Marker Requirements

The "ideal" plasma marker to be used for evaluation of myocardial injury in patients presenting with chest pain suggestive of acute coronary syndromes in primary or secondary care would need to meet three criteria, i.e., (i) show absolute myocardial specificity and (ii) be instantaneously released into the circulation upon myocardial injury, while (iii) a test should be available that allows the accurate and rapid (minutes) assessment of its elevated concentration in plasma so as to permit the use of the test result in triaging of the patient. Unfortunately, such "ideal" marker does not exist (Gravning and Kjekshus 2008):

i. *Cardiac specificity.* Although the troponins show virtually absolute cardiac specificity and therefore have been adopted as primary marker for ACS diagnostics to be included in both the US and European guidelines for the management of ACS (Hamm et al. 2011), they appear in plasma only 3–8 h after onset of myocardial injury which in a substantial number of cases is too late to influence the initial triaging process. Hence, in cases where MI is not revealed on ECG, echocardiogram, or other imaging techniques, patients will be monitored up to 9–12 h to rule in or rule out an MI based on an elevated plasma troponin. For

those patients that turn out not to have MI, the latter procedure involves a marked financial burden that should be avoided when possible.

ii. *Early release into plasma*. H-FABP is the earliest marker to be elevated in plasma following myocardial injury and yet does not show absolute cardiac specificity because an elevation of plasma H-FABP could also be due to skeletal muscle injury. However, in view of the presence of H-FABP only in red (oxidative) skeletal muscle fibers and only in minute amounts (i.e., <5 % of that in the myocardium), a significant release of H-FABP from skeletal muscle takes place merely in specific cases such as eccentric exercise (Sorichter et al. 1998). In addition, in reported studies on the use of H-FABP for MI diagnostics to our knowledge, no such cases have been described in which plasma H-FABP was falsely elevated due to skeletal muscle injury.

iii. *Rapid test result*. The availability of an appropriate test that allows the rapid assessment of elevated marker concentrations in plasma is crucial to aid in the diagnosis of MI. Because H-FABP nor the troponins show enzymatic activity, such test has to be based on immunochemical detection of the protein. For both markers tests are available for use in the hospital emergency room or chest pain unit. For instance, Randox has developed a turbidimetric H-FABP assay that provides a quantitative result with a range of 2.5–120 ng/ml in serum in 14 min (see Table 2). Tests for cTnT and cTnI have been developed and marketed by most diagnostic companies, yet not all provide a high-sensitive test that complies with the requirements of current standard definition (Thygesen et al. 2012). To meet these requirements, high-sensitivity or ultrasensitive troponin assays (hs-cTnT and hs-cTnI) are needed. The limit of detection of these assays is 10–100-fold lower than that of the conventional troponin assays. This suggests their application for diagnosing smaller MIs otherwise undetected or for identifying MI earlier when abnormal troponin levels are below detection by conventional assays. Recently, PoC tests have become available for both H-FABP and the troponins; these will be discussed in a separate paragraph.

Heart-Type Fatty Acid-Binding Protein as Plasma Marker of Cardiac Injury

The cytoplasmic protein FABP has a relatively small size (14.5 kDa) and functions as an intracellular fatty acid carrier in parenchymal cells, thus supplying essential substrates for energy production in the myocytes (Glatz and Van der Vusse 1990). It comprises as much as 1–2 % of total cardiac cytosolic proteins, making it one of the most abundant cytosolic proteins. H-FABP is also found in small amounts in (slow-twitch oxidative) skeletal muscle, in distal tubule cells of the kidney, and in some parts of the brain (Schaap et al. 1998). The potential of H-FABP to be used as plasma marker of myocardial injury was suggested first in 1988 (Glatz et al. 1988). Its cytosolic occurrence, cardiac tissue abundance, and small size make that, upon myocardial cellular damage, H-FABP is released rapidly and in appreciable amounts to the interstitial space, from where it escapes through the endothelial clefts into the

Table 2 Overview of H-FABP assays in plasma

H-FABP assay	Test principle	Sample type	Detection limit (ng/ml)	Time to result (min)	Regulatory status (RUO/CE)	Reference
Laboratory immunoassays						
Randox Evidence Investigator Cardiac Array	Biochip	Serum/plasma	0.15	20	CE	www.randox.com
Randox immunoturbidimetric H-FABP	Turbidimetry	Serum/plasma	0.75	14	CE	www.randox.com
Roche Diagnostics H-FABP	Turbidimetry	Serum/plasma	1.1	8	NR	
Markit-M H-FABP (Dainippon Pharmaceutical)	ELISA	Serum/plasma	1.25	75	RUO	www.bmassay.com
Hycult Biotechnology H-FABP	ELISA	Serum/ plasma/urine	0.1	50–120	RUO	www.hycultbiotech.com
Oxis Research H-FABP	ELISA	Serum	NR	90	RUO	www.oxisresearch.com
Point-of-care (bedside) tests[a]						
Rapicheck (Dainippon Pharmaceutical)	Lateral flow	Whole blood	6.2	15	CE	www.bmassay.com
CardioDetect (8sens.biognostic)	Lateral flow	Whole blood	7	15–20	CE	www.biognostic.de
QuickSens H-FABP (8sens. biognostic)	Lateral flow	Plasma/whole blood	0.6	15–20	CE	www.biognostic.de
H-FABP True Rapid Test (FABPulous)	Lateral flow	Whole blood	4	5	CE	www.fabpulous.com

Several characteristics (test principle, sample type, detection limit, time to result, regulatory status, and reference) of currently available point-of-care and laboratory assays for testing heart-type fatty acid-binding protein

Abbreviations: *CE* European conformity quality mark, *H-FABP* heart-type fatty acid-binding protein, *NR* not reported, *RUO* research use only

[a]Rapicheck, CardioDetect, and H-FABP True Rapid Test are qualitative tests; QuickSens H-FABP uses a reader providing a quantitative test result

vascular space. While larger cytosolic proteins such as CK-MB (86 kDa, i.e., five times larger than H-FABP) appear in the interstitium simultaneously with H-FABP, these larger proteins are delayed in their plasma appearance because the speed of reaching the plasma compartment is governed by the permeability of the endothelial barrier (which is dependent on protein size) and by lymph drainage (Van Nieuwenhoven et al. 1996). The troponins also appear in plasma markedly later than H-FABP, despite their relative small size (troponin T, 37 kDa, i.e., three times larger than H-FABP; troponin I, 22 kDa, i.e., 1.5 times larger than H-FABP). This is due to the fact that following cellular damage the troponins first need to be proteolytically cleaved from the contractile matrix. As a result, H-FABP is the earliest available plasma marker of cardiac injury (Glatz 1998; Glatz et al. 2002). Release of H-FABP from injured myocardium is essentially complete, indicating that infarct size can be estimated from the cumulative release of H-FABP into plasma (Glatz et al. 1994; De Groot et al. 1999). Because the subsequent clearance of H-FABP from plasma occurs via the kidneys, renal insufficiency could hamper such estimation (Wodzig et al. 1997b). Renal clearance of small proteins such as H-FABP is rapid and thus contributes markedly to maintaining a relatively low plasma reference concentration. In apparently healthy subjects, the plasma H-FABP concentration is between 1 and 2 ng/ml (Pelsers et al. 1999; Pagani et al. 2002; Niizeki et al. 2007; Bathia et al. 2009; Glatz and Mohren 2013), which is only <0.0001 % of the tissue content (estimated at 170 μmol/L (Vork et al. 1993) which is equivalent to 2500 μg/ml). As a result, there is a steep gradient of H-FABP from myocardial cells to plasma which adds to the high sensitivity of this marker for tissue injury detection. Circulating levels of H-FABP are somewhat higher in males (ca. 1.9 ng/ml) than in females (ca. 1.5 ng/ml) and slightly increase with age, especially after 50 years, which most likely is explained by the decrease in renal function in elderly people (Wodzig et al. 1997a; Glatz and Mohren 2013).

H-FABP and Troponin are Sensitive Markers of Myocardial Tissue Injury

Representative mean plasma release curves of H-FABP and cTnT and, for comparison, myoglobin are shown in Fig. 1. These curves were recorded for 15 patients with MI, treated with reperfusion therapy, from whom blood samples were obtained frequently during the first 24 h of hospitalization (Glatz et al. 2002; Pelsers et al. 2005). Peak plasma concentrations of FABP and myoglobin are reached at about 4 h after onset of symptoms, whereas for cTnT this takes about 15 h (see Fig. 1) and for CK-MB about 12 h (data not shown). Plasma FABP and myoglobin return to their respective reference values already within 24 h after MI, indicating the usefulness of both markers particularly for the assessment of a recurrent infarction (Van Nieuwenhoven et al. 1995) which might be missed by CK-MB or the troponins as these markers return much slower to their normal plasma value. Importantly, for MI patients not treated with thrombolytics, H-FABP peaks after approximately 8 h and remains elevated up to 24–36 h after chest pain onset (Van Nieuwenhoven

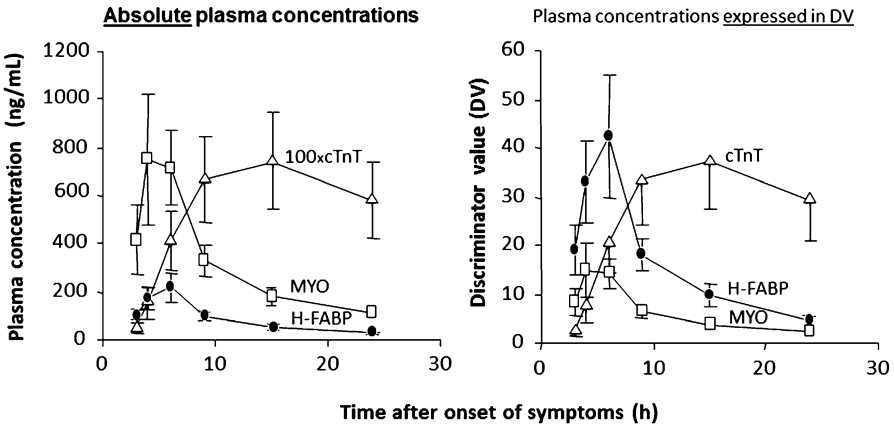

Fig. 1 Plasma release curves for three cardiac marker proteins. Mean plasma concentrations of heart-type fatty acid-binding protein (H-FABP) (●), myoglobin (MYO) (□) and cardiac troponin T (cTnT) (Δ) as a function of time after acute myocardial infarction for 15 patients who were treated successfully with reperfusion therapy and from whom serial blood samples were obtained up to 24 h after onset of symptoms. The data are presented as plasma concentrations in ng/mL (*left panel*) or relative to the upper reference limit for H-FABP (6 ng/mL), MYO (60 ng/mL) and cTnT (0.1 ng/mL) (*right panel*). Data refer to mean ± S.E.M (Adapted from Glatz et al. (2002), with permission). Abbreviations: *cTnT* cardiac troponin T, *DV* discriminator value, *h* hours, *H-FABP* heart-type fatty acid-binding protein, *MYO* myoglobin

et al. 1995). This latter finding indicates that the so-called diagnostic window of H-FABP for detection of myocardial injury in patients presenting with chest pain stretches to 24–36 h after onset of symptoms.

When expressed relative to the upper reference limit (or discriminator value) of each marker protein, it is clear that the rise in plasma concentrations is highest for H-FABP, closely followed by cTnT, and is much lower for myoglobin (see Fig. 1, right panel). This difference is explained mainly by the markedly lower relative plasma reference concentrations of H-FABP and cTnT when compared to myoglobin. Taken together, the sensitivity of H-FABP and cTnT for cardiac injury detection markedly outperforms that of myoglobin (see Fig. 1), as well as that of CK-MB (data not shown).

In more recent years, the performance of H-FABP for acute MI diagnosis has been centered on its comparison with cTnT/cTnI and/or hs-cTnT/hs-cTnI, thereby focusing on early exclusion of MI. Table 3 lists the larger and more recent clinical studies that have directly compared H-FABP and troponin applying quantitative assays that are currently in use. Quantitative tests are independent of the cutoff level that is chosen and thus allow a proper evaluation of the markers. In contrast, the performance of a qualitative test (such as a PoC test) depends on the assigned cutoff (see discussion below).

The emerging overall picture is that the area under the receiver operating characteristic (ROC) curve (AUC) for H-FABP is similar to that for the conventionally analyzed troponins (see Table 3, upper part). However, when analyzed with high-

Table 3 Diagnostic performance of heart-type fatty acid-binding protein (H-FABP) and cardiac troponin (cTn) for acute myocardial infarction

Reference	Admission time[a] (h)	H-FABP				cTn (cTnT or cTnI)			
		Cutoff (ng/ml)	AUC	Sensitivity	Specificity	Cutoff (ng/ml)	AUC	Sensitivity	Specificity
Conventional cTn tests									
Mion et al. 2007 ($n = 132$)	3.8 (FR 1.5–13)	5.8	0.92	0.83	0.93	0.032 cTnI	0.75	0.55	0.98
McCann et al. 2008 ($n = 415$)	0–4	5.0	0.77	0.73	0.71	0.03 cTnT	0.78	0.55	0.95
	5.3 (IQR 2.7–8.9)		0.74	0.76	0.61		0.88	0.75	0.94
Haltern et al. 2010 ($n = 94$)	0–4	7.3	0.76	0.86	0.66	0.03 cTnT	0.71	0.42	1.00
	4 (CI 3–6)		0.71	0.71	0.65		0.87	0.74	1.00
Alhadi and Fox 2010 ($n = 100$)	<6	5.0	NR	0.80	0.92	0.032 cTnI	NR	0.56	0.81
Gururajan et al. 2010 ($n = 485$)	<6	17.7	0.97	0.87	0.93	0.032 cTnI	0.77	0.54	0.95
Kurz et al. 2011 ($n = 94$)	6.0 (IQR 2.5–15)	6	0.81	0.89	0.62	0.03 cTnT	0.72	NR	NR
Body et al. 2011	3.5 (IQR 1.8–7)	58	0.86	0.75	0.89	0.055 cTnI	0.70	0.42	0.96
Keller et al. 2011 ($n = 1{,}818$)	4.3 (IQR 2.0–13)	5.8	0.89	NR	NR	0.032 cTnI	0.92	0.79	0.95
McMahon et al. 2012 ($n = 1{,}128$)	0–3	5.2	0.84	0.64	0.84	0.037 cTnT	0.76	0.50	0.93
	3–6		0.89	0.85	0.89		0.85	0.68	0.94
	6–12		0.94	0.90	0.94		0.90	0.81	0.94
	12–24		0.97	0.90	0.91		0.98	0.96	0.94
	24–48		0.91	0.63	0.91		0.98	0.97	0.95
	>48		0.87	0.66	0.91		0.94	0.88	0.94
Ruff et al. 2013 ($n = 343$)	8.7 (IQR 5–14)	5	0.78	0.63	0.79	0.10 cTnI	0.91	0.77	0.97
High-sensitive (hs-)cTn tests									
Kurz et al. 2011 ($n = 94$)	6.0 (IQR 2.5–15)	6	0.81	0.89	0.62	0.014 hs-cTnT	0.82	0.82	0.76

Keller et al. 2011 ($n = 1{,}818$)	4.3 (IQR 2.0–13)	5.8	0.89	NR	NR	0.030 hs-cTnI	0.99	0.82	0.92
Eggers et al. 2012 ($n = 360$)[b]	<8	5.8	0.71	0.39	0.95	0.014 hs-cTnT	0.74	0.79	0.75
Kagawa et al. 2013 ($n = 114$)	NR	6.2	0.59	0.78	0.22	0.028 hs-cTnI	0.89	0.81	0.79
Reiter et al. 2013 ($n = 1{,}074$)[b]	<3	4.2	0.85	NR	NR	0.014 hs-cTnT	0.92	NR	NR
	<12		0.84	0.72	0.80		0.94	0.93	0.77
Ruff et al. 2013 ($n = 343$)	8.7 (IQR 5–14)	5	0.78	0.63	0.79	0.040 hs-cTnI	0.96	0.92	0.92
Cappellini et al. 2013 ($n = 67$)	<8	3.5	0.84	1.00	0.39	0.014 hs-cTnT	0.81	0.81	0.56
Collinson et al. 2014 (Heart) ($n = 838$)	3.7 (IQR 2.6–5.8)	3	0.84	0.65	0.94	0.040 hs-cTnT	0.92	0.78	0.96
Bank et al. 2015 (ACB) ($n = 453$)	3.0 (IQR 1.8–6.8)	7	0.73	0.54	0.81	0.014 hs-cTnT	0.88	0.71	0.90
Jacobs et al. 2015 (ACB) ($n = 584$)	3.0 (IQR 1.8–5.1)	4	0.81	0.60	0.86	0.045 hs-cTnI	0.88	0.68	0.96

Comparison of recent clinical trials using quantitative tests of H-FABP and cTn and acute myocardial infarction as an outcome. Cutoff level (being the 99th percentile upper reference level used), area under the curve, sensitivity, and specificity are given for both H-FABP and cTn

Abbreviations: *AUC* area under receiver operating characteristic (ROC) curve, *CI* 95 % confidence interval, *cTn* cardiac-specific troponin, *cTnI* cardiac-specific troponin I, *cTnT* cardiac-specific troponin T, *FR* full range, *h* hours, *H-FABP* heart-type fatty acid-binding protein, *hs-cTnI* high-sensitive cardiac-specific troponin I, *hs-cTnT* high-sensitive cardiac-specific troponin T, *IQR* interquartile range, *non-STEMI* non-ST-elevated myocardial infarction, *NR* not reported

[a]Median time from symptom onset to admission, with full range (FR), 95 % confidence interval (CI), or interquartile range (IQR)

[b]Non-STEMI patients only

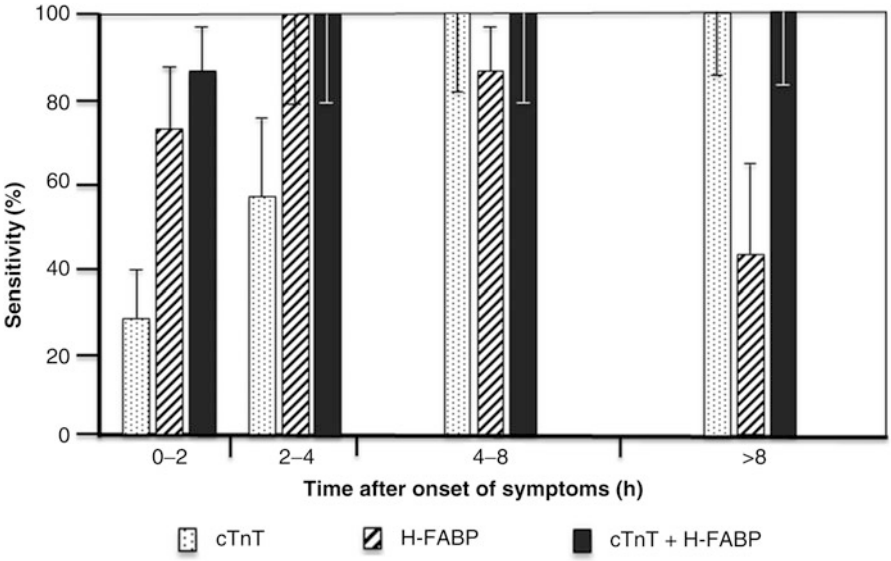

Fig. 2 Diagnostic performance of plasma heart-type fatty acid-binding protein (H-FABP) and cardiac troponin T (cTnT) as a function of time after onset of symptoms suggestive of acute coronary syndrome. Sensitivities for myocardial infarction of the markers separately and a combined approach (either one positive) are presented with varying symptom durations (Reprinted from Haltern et al. (2010), Copyright (2010), with permission). Abbreviations: *cTnT* cardiac troponin T, *h* hours, *H-FABP* heart-type fatty acid-binding protein

sensitivity assays, troponin exhibits a significantly greater AUC than H-FABP (see Table 3, lower part). Irrespective of the assay format used, the overall specificity is higher for troponin than for H-FABP; however the overall sensitivity is lower for troponin than for H-FABP. When distinction is made for patients seen early after onset of symptoms (e.g., within 3–4 h) versus patients admitted to the emergency room at a later point in time, the performance of H-FABP is significantly better in the first hours after MI (McCann et al. 2008; Haltern et al. 2010; McMahon et al. 2012; Reiter et al. 2013). This finding is illustrated in the report by Haltern et al. (2010), who evaluated patient groups according to symptom duration (see Fig. 2). Sensitivity of H-FABP at presentation was > twofold higher than that of conventional cTnT when symptom duration was <2 h and increased to 100 % in the group with symptom duration of 2–4 h. In this latter group, the sensitivity of cTnT was only 55 %. For patients admitted >4 h, the sensitivity of the two markers switched: the sensitivity of cTnT reached 100 %, while that of H-FABP decreased significantly (see Fig. 2). The data reported by McMahon et al. (2012) (see Table 3) reveal a similar bell-shaped curve for the sensitivity of H-FABP as a function of the admission delay. The corollary is that combining H-FABP and cTnT (i.e., either marker elevated) provides a significant improvement in sensitivity for patients presenting <4 h after symptom onset while being maintained at 100 % for patients presenting >4 h (see Fig. 2). These data indicate the usefulness of combining H-FABP and

troponin for improved early diagnosis of ACS. In conclusion, each of the biomarkers has its own characteristics, with H-FABP being the preferred marker to diagnose AMI in the early hours after onset of symptoms and (hs-)cTnT or (hs-)cTnI the preferred marker from 3 to 4 h onward after presentation.

Combining H-FABP and Troponin for Ruling Out ACS

As discussed above, especially in the early hours after onset of symptoms, H-FABP shows a superior sensitivity to troponin, even when high-sensitivity troponin tests are used. However, published data indicate that measurement of H-FABP alone cannot enable a safe rule-out of AMI at presentation, i.e., NPV >97–98 % (see Table 3 and references therein). This is illustrated by the results of a meta-analysis of 16 studies including 3,709 patients with suspected AMI, which reported for H-FABP a pooled sensitivity of 84 % (95 % confidence interval (CI) 76–90 %) and a pooled specificity of 84 % (95 % CI 76–89 %) (Bruins Slot et al. 2010). As mentioned above, combining H-FABP and cardiac-specific troponin (cTn) significantly improves the diagnostic sensitivity (see Fig. 2), especially when using hs-cTnT or hs-cTnI assays. A systematic review by Carroll et al. (2013) on four clinical studies (total of 1,598 patients) on combinations of quantitatively assessed H-FABP and cTn versus cTn alone at presentation (Mion et al. 2007; McCann et al. 2008; Haltern et al. 2010; Body et al. 2011) revealed that the addition of H-FABP to cTn increased sensitivity from 42–75 % to 76–97 % but decreased specificity from 95–100 % to 65–93 %. In a subsequent review, Lippi et al. (2013) analyzed eight studies (totaling 2,735 patients), including four studies applying qualitative H-FABP tests, to observe that the addition of H-FABP to cTn increased pooled sensitivity from 73 % to 91 % which however was counterbalanced by a decreased pooled specificity from 94 % to 82 %. These reviews did not include the earlier extensive study (1,818 patients) described by Keller et al. (2011), combining quantitative H-FABP and hs-cTnI. These investigators reported that the addition of H-FABP to hs-cTnI increased sensitivity from 73 % to 85 %, decreased specificity from 95 % to 91 %, and decreased positive predictive value (PPV) from 66 % to 60 % but increased the NPV from 95.9 % to 97.6 % (Keller et al. 2011). The latter indicates that the NPV of the combined test fulfills the diagnostic requirements for application as a rule-out parameter. In this study, the time between chest pain onset and admission to the emergency room (first blood sample) was 4.3 h (range 2.0–13 h). It was not examined whether the performance of the combined markers is dependent on the time of presentation of the patient.

In a study by Ruff et al. (2013), similar data were found. The addition of H-FABP to a conventional cTnI assay significantly enhanced both the sensitivity (from 77 % to 92 %) and NPV (from 92 % to 97 %) of MI diagnosis. When H-FABP was added to hs-cTnI, the overall diagnostic accuracy was not improved when compared to the performance of hs-cTnI alone, but in early presenters (<6 h after onset of symptoms) the combination did improve both sensitivity and NPV (each to 100 %) (Ruff et al. 2013). In contrast, in the recent study by Reiter et al. (2013), no synergistic

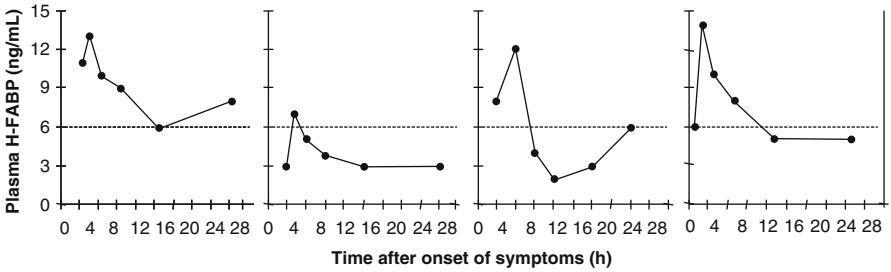

Fig. 3 Plasma release curves of heart-type fatty acid-binding protein (H-FABP) for patients with unstable angina pectoris. Examples of individual patients clinically diagnosed as having unstable angina pectoris. In each case plasma H-FABP was elevated above its discriminator value (of 6 ng/mL; *dashed line*) and shows a typical "rise and fall" pattern suggesting the occurrence of minor myocardial injury. Data obtained from the *EuroCardi* multicenter trial (Adapted from Glatz et al. (2002), with permission). Abbreviations: *h* hours, *H-FABP* heart-type fatty acid-binding protein

performance of H-FABP and hs-cTnT was reported, but in this study patients with ST-segment elevation in the initial ECG were excluded.

Minimal Myocardial Injury

Patients with a clinical diagnosis of UAP often show an elevated plasma concentration of H-FABP (Katrukha et al. 1999; Valle et al. 2008). Analysis of serial plasma samples after onset of symptoms has revealed that, also in these patients, there is a characteristic rise and fall of plasma markers reminiscent of their release from injured myocardium (see Fig. 3; Glatz et al. 2002). This reflects the sensitivity of the marker H-FABP for myocardial cell injury and should not be labeled as false positive. Patients with such minimal (or minor) myocardial injury – also referred to as subclinical myocardial injury – may have a prognosis as serious as do patients with definite MI (Hamm et al. 1992) and therefore may benefit from similar medical treatment. Interestingly, H-FABP has also been applied as plasma marker to identify minimal myocardial injury in nonalcoholic fatty liver disease (Basar et al. 2013).

H-FABP and Kidney Function

H-FABP is cleared by glomerular filtration in the kidneys, and thus, H-FABP values can be elevated in case of severe kidney damage (eGFR <30 ml/min). After myocardial injury, H-FABP is eliminated by renal clearance and values return to normal after 24–36 h. Therefore, it can be used up to 24 h after onset of complaints.

Primary Care: Extension of Diagnostic Strategies in ACS and Potential Role of H-FABP

Because in primary care the median period between onset of symptoms of MI and diagnostic assessment by the GP in most countries is 2–3 h (Hooghoudt et al. 1998) and only in rural areas will be longer, the troponins cannot be used as the lead parameter for stratification of patients. Furthermore, as stated above, although troponin levels are of high importance in ruling out AMI in a secondary care setting, unacceptable practical limitations are faced in using troponin in a primary care setting. Therefore, attention is drawn to alternative biomarkers. Of the biomarkers studied to date, H-FABP is placed among the earliest of plasma markers (Dekker et al. 2010). In case of AMI, elevation of plasma H-FABP can be detected within the first 1–2 h after onset of complaints (Glatz 1998; Mad et al. 2007). Venous levels are increased to concentrations up to 40-fold the normal concentration (Pelsers et al. 2005). Especially in cases of AMI, H-FABP levels correspond impressively to hs-cTnT levels (Willemsen et al. 2015). Therefore, H-FABP may have meaningful potential in improving the triage of patients suspected of AMI.

The main requirement for any biomarker test in primary care is the possibility to measure and obtain a result within several minutes at the point of care, combined with a potency to rule out AMI with a high NPV that should be >97–98 %. The NPV largely depends on sensitivity of the test and prevalence of the disease and less on specificity. At this moment, studies reviewing early PoC markers are characterized by methodological imperfections (Bruins Slot et al. 2013a). The function of H-FABP and other early markers combined with signs and symptoms in risk classification in a low-prevalence setting such as primary care is still to be determined (Than et al. 2011; Tomonaga et al. 2011; Reiter et al. 2013). When H-FABP testing is combined with signs and symptoms in a *diagnostic algorithm*, NPV hypothetically improves, and thus the number of patients that are referred by a GP, but turn out to have no ACS, could be reduced. Even with a moderate amount of false-positive results, such an algorithm could improve daily practice since currently the majority of patients without underlying ACS are referred to secondary care facilities.

A primary care study evaluating a PoC test on H-FABP did not lead to implementation of PoC testing in daily practice. Limiting test characteristics in this study were insufficient sensitivity (using a test cutoff point for H-FABP of 7 ng/ml), robustness (11 % invalid results), and a time to result of 15–20 min that is considered too long for acute situations in general practice. However, the PoC device for H-FABP used in this study used a cutoff value of 7 ng/ml, which is above the 99th percentile of 5.7 ng/ml as found in a normal reference population (Glatz and Mohren 2013). Retrospective measurement of plasma H-FABP values revealed added value of H-FABP, although insufficient to reach a NPV of 98 % or more.

Reported 99th percentile values for H-FABP range from 5.2 to 7.3 ng/ml (Pelsers et al. 1999; Pagani et al. 2002; Niizeki et al. 2007; Bathia et al. 2009; Glatz and Mohren 2013; Haltern et al. 2010). However, when derived from ROC curves, optimal cutoff levels for H-FABP at presentation to discriminate AMI from other

Table 4 Diagnostic values of H-FABP and hs-cTnT at different cutoff points

Biomarker	Cutoff value	Sensitivity	Specificity	Expected NPV in primary care, with a prevalence of AMI of 17 %
H-FABP	4 ng/ml	0–3 h 56,1 %	0–3 h 67,5 %	0–3 h 88,3 %
		3–24 h 91,5 %	3–24 h 80,7 %	3–24 h 97,9 %
hs-cTnT	14 ng/ml	0–3 h 56,3 %	0–3 h 70 %	0–3 h 88,7 %
		3–24 h 91,5 %	3–24 h 68,4 %	3–24 h 97,5 %
H-FABP	7 ng/ml	0–3 h 20,8 %	0–3 h 95 %	0–3 h 85,4 %
		3–24 h 76,3 %	3–24 h 91,2 %	3–24 h 94,9 %
hs-cTnT	50 ng/ml	0–3 h 14,6 %	0–3 h 92,5 %	0–3 h 84,1 %
		3–24 h 78 %	3–24 h 94,7 %	3–24 h 95,5 %
hs-cTnT	100 ng/ml	0–3 h 8,3 %	0–3 h 100 %	0–3 h 84,2 %
		3–24 h 72,9 %	3–24 h 98,2 %	3–24 h 94,6 %

Sensitivity, specificity, and negative predictive value (NPV) for acute myocardial infarction of H-FABP and hs-cTnT at different cutoff points are given. Patients with an estimated glomerular filtration rate below 30 ml/min were excluded. NPV is calculated using a prevalence of AMI of 17 %, as has been reported among patients presenting with chest pain in primary care (Willemsen et al. 2015 extended data)

AMI acute myocardial infarction, *h* hours, *H-FABP* heart-type fatty acid-binding protein, *hs-cTnT* high-sensitive cardiac-specific troponin T, *NPV* negative predictive value

causes of chest pain generally are lower, ranging from 3.3 ng/ml (Freund et al. 2012) to 4.4 ng/ml (Reiter et al. 2013). Similarly, in a recent study of 218 consecutive patients with new-onset chest pain seen by a GP, the ROC-derived optimal cutoff for H-FABP was 4.0 ng/ml (Willemsen et al. 2015). Improved diagnostic performance of H-FABP using such lower values has been documented and advocated (Ruff et al. 2013; Cappellini et al. 2013; Carroll et al. 2013).

As a consequence, recently, a new PoC H-FABP test was designed to overcome the earlier mentioned limitations, by lowering the cutoff value to 4 ng/ml in a secondary care population, where 50 % of patients were diagnosed with AMI (Willemsen et al. 2015). This is below the 99th percentile of H-FABP – in a normal reference population determined by the manufacturer in a healthy reference population of blood donors between 40 and 70 years of age (Glatz and Mohren 2013). Setting the cutoff value at 4 ng/ml leads to a diagnostic performance equaling that of hs-cTn. Thus, H-FABP has the same properties as hs-cTn with the generally used cutoff value of 14 ng/ml for high-sensitive troponin T, used as gold standard for AMI, whereas the earlier used PoC H-FABP test with a cutoff point of 7 ng/ml correlates to high-sensitive troponin T with a cutoff point of 50 ng/ml (see Table 4).

Calculated NPV in a primary care population (with an incidence of ACS of 20 % or less) would reach 88.3 % in patients with a duration of complaints of less than 3 h and 97.9 % in patients with a duration of complaints of 3–24 h. Currently this PoC H-FABP test is studied in primary care (Willemsen et al. 2014). At the cutoff point of 4 ng/ml, this PoC H-FABP test is regarded as positive by its users in 95 % of cases, and coefficient of variance (CV) is <10 %. Furthermore, decrease of invalid results

to an amount of less than 2 % has improved robustness, and a time to result of 5 min increases usability in an acute setting.

Secondary Care: Extension of Diagnostic Strategies in ACS and Potential Role of H-FABP

In secondary care, where safe rule-out based on one measurement would be preferable above several measurements with a given time interval, ongoing studies focus on the potency of hs-cTn as well as the potency of other biomarkers to be combined with a single hs-cTn measurement. cTnT or cTnI measurement has become the cornerstone of diagnosing MI in secondary care (Hamm et al. 2011; Thygesen et al. 2012). Adding copeptin or H-FABP to troponin in an early phase in emergency room settings increases sensitivity for ACS, but so far the combination has failed to safely rule out ACS in an early stage (Body et al. 2011; Charpentier et al. 2011). Until recently, troponin assays have gained sensitivity due to usage of highly sensitive techniques (resulting in hs-cTn measurements). The additional value of H-FABP testing besides hs-cTn in some studies is small or unclear (Carroll et al. 2013; Lippi et al. 2013; Vaidya et al. 2014; Bank et al. 2015). Several studies however have described an added value of H-FABP when measured besides troponin in an emergency room setting in an early phase (McMahon et al. 2012; Carroll et al. 2013; Gami et al. 2015; Jacobs et al. 2015). As a solitary rule-out test at admission, hs-cTnT outperforms H-FABP slightly, but H-FABP tested in addition to hs-cTnT leads to an increase of sensitivity compared to hs-cTnT alone (Collinson et al. 2014). Recently, promising results were published of hs-cTn measurement combined with H-FABP measurement, ECG findings, and several clinical findings in early rule-out of severe underlying disease in patients presenting with chest pain (Body et al. 2014a, b).

Concluding Remarks

Twenty-five years after the first report on the potential use of H-FABP as a plasma biomarker for myocardial injury (Glatz et al. 1988), a large number of clinical studies performed by a variety of researchers applying a multitude of assay formats have now documented that H-FABP (i) is rapidly released from injured myocardium to be the earliest available plasma marker after an ischemic insult; (ii) shows a sensitivity for cardiac injury detection that is similar to that of cTn and markedly better than that of all other known cardiac marker proteins; (iii) for AMI diagnosis or exclusion shows added value on top of the recommended markers cTnT or cTnI, even when these are determined by high-sensitivity assays, with the added value being larger for patients presenting early (<4 h) after onset of symptoms; and (iv) in early (<4 h) presenters may be suited as stand-alone diagnostic test for safely ruling out AMI. The latter is relevant especially for primary healthcare and would markedly increase cost-effectiveness of AMI diagnosis but awaits appropriate prospective trials.

H-FABP also is established to be a robust early predictor of future cardiovascular events or mortality independent of other cardiac risk factors including plasma troponin. As a result, the clinical utility of H-FABP both as early marker for the evaluation of suspected ACS and as prognostic marker is undisputed, especially when it is part of a diagnostic assessment combining several early findings (Pelsers et al. 2005; Viswanathan et al. 2012; Body 2012; Lackner 2013; Renneberg et al. 2013; Body et al. 2014b).

Future Perspective

Despite the strong data available for H-FABP on its performance as biomarker for early triaging of patients with chest pain, FABP has not (yet) gained widespread use. Likely this will change in the near future, when more PoC tests (that employ optimal cutoff levels) and tests for clinical chemistry analyzers will become available. This would hold especially for tests that would give results within the time of a typical primary care consultation of 7–10 min. H-FABP then may be adopted as "early" plasma marker to be applied alone or beside a "late" marker such as cTnT or cTnI. In emergency care diagnostics, the future focus will be on very early exclusion of AMI. H-FABP could be excellently suited for this purpose especially when measured in combination with several other early findings in patients presenting with chest pain. In this way, a major reduction of costs otherwise spent on hospitalization and extensive diagnostic follow-up of non-AMI patients is enabled (Body et al. 2014a,b). Given the large numbers of patients who present with chest pain (for instance, in Germany >750,000 annually) (Nilsson et al. 2003), due to its diagnostic accuracy and due to the reliability of contemporary PoC tests, H-FABP may well become part of a new golden standard for improving quality yet reducing cost of care.

Potential Applications to Prognosis and Other Diseases or Conditions

Besides its (future) use as a biomarker for acute ischemic heart disease, H-FABP has been reported to be valuable as a prognostic marker to assess future risks on major cardiac events in patients.

H-FABP is an early and independent predictor of future cardiovascular events and thus may help to improve long-term risk stratification of patients with acute chest pain (O'Donoghue et al. 2006; Kilcullen et al. 2007; McCann et al. 2009; Garcia-Valdecasas et al. 2011; Viswanathan et al. 2010). Increased plasma H-FABP is a robust predictor of major cardiac events (such as death or MI) within 2 years in patients with chest pain and remained significant in a multivariate analysis that included both various plasma biomarkers and echocardiographic assessment of cardiac morphology and function (O'Donoghue et al. 2006; Kilcullen et al. 2007; McCann et al. 2009; Reiter et al. 2013). The

NPV regarding 1-year and 2-year mortality was 99 % (CI 98–100) and 98 % (CI 96–99), respectively, for plasma H-FABP <2.7 ng/ml (Ruff et al. 2013). H-FABP plasma concentration identifies patients at risk for death and major cardiac events even when troponin and/or NT-proBNP are not elevated (O'Donoghue et al. 2006; Kilcullen et al. 2007; Reiter et al. 2013). These findings are consistent when H-FABP is compared to troponin values obtained with hs-cTnT assays (Reiter et al. 2013; Viswanathan et al. 2010). These findings confirm H-FABP to be a rapidly released and sensitive biomarker of minor myocardial injury as caused by ongoing and recurrent myocardial ischemia.

Similarly, in patients with congestive heart failure, plasma H-FABP identifies those at high risk for future cardiac events, independent of cTnT (Niizeki et al. 2008; Kutsuzawa et al. 2012). In patients undergoing coronary artery bypass grafting (CABG), H-FABP is a superior independent predictor of postoperative mortality and ventricular dysfunction (Muehlschlegel et al. 2010). H-FABP appears a promising early biomarker also for risk stratification of normotensive patients with acute pulmonary embolism and was found to perform markedly better than either plasma cTnT or right ventricular dysfunction (Kaczynska et al. 2006; Puls et al. 2007; Dellas et al. 2010; Boscheri et al. 2010). In case of a negative H-FABP test, these patients had an excellent prognosis regardless of echocardiographic findings, while patients with an elevated plasma H-FABP had a complication rate of 23 %. In sepsis, H-FABP appears to be an independent prognostic factor for 28-day mortality (Jo et al. 2012; Zhang et al. 2012). Finally, an elevated H-FABP concentration measured during the follow-up of MI (median of 20 days post-MI) predicted long-term all-cause mortality and readmission for heart failure significantly better than did plasma cTnT, for a time interval up to 5 years post-MI (Matsumoto et al. 2013).

In conclusion, when plasma H-FABP is elevated, a decreased clinical outcome can be expected in patients with chest pain, congestive heart failure, and pulmonary embolism, in patients after CABG, and in post-MI patients.

Summary Points

- An increased number of patients present with chest pain of unknown cause. There is a need for additional diagnostic tools to facilitate a cost-effective strategy for these patients.
- Plasma marker proteins of myocardial injury have become the most reliable parameter for diagnosis of patients with chest pain.
- The small but abundant myocardial heart-type fatty acid-binding protein (H-FABP) appears a promising plasma marker for myocardial injury detection.
- Among plasma marker proteins, H-FABP and troponin display the highest sensitivity for detection of myocardial injury.
- Subclinical myocardial injury was found to result in elevated plasma H-FABP concentrations.
- No plasma marker exists that is ideally suited for myocardial injury detection.

- Because of its more rapid release from injured myocardium when compared to troponin, H-FABP is applicable in particular for early monitoring of myocardial injury.
- A multimarker approach, i.e., combining H-FABP and troponin, markedly improves diagnostic performance of the markers when compared to each of the markers alone.
- Because point-of-care tests with low cutoff values between positive and negative are available for H-FABP, H-FABP is a promising biomarker for ruling out myocardial infarction in this setting.
- Several point-of-care tests for H-FABP have been described and are expected to facilitate diagnosing patients with chest pain especially in primary care.
- Further research in primary care and early after presentation in secondary care is needed to define the future role of H-FABP in ruling out acute coronary syndrome in patients with chest pain of unknown cause.

Acknowledgments Jan Glatz is currently the CSO of FABPulous BV.

Geert-Jan Dinant and Robert Willemsen are currently involved in research independently supported by a grant from FABPulous BV.

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Uncarboxylated Matrix Gla Protein as a Biomarker in Cardiovascular Disease: Applications for Research and for Routine Diagnostics

12

Cees Vermeer, Nadja E. A. Drummen, Marjo H. J. Knapen, and Fokko J. Zandbergen

Contents

Abstract

Matrix Gla protein (MGP) is a vitamin K-dependent protein acting as an inhibitor of vascular calcification. Poor dietary vitamin K intake results in the formation of inactive MGP, also known as uncarboxylated MGP, which is set free in the blood stream where it is available for quantification by ELISA-based assays. In the healthy adult population, significant concentrations of uncarboxylated MGP are found, suggesting a widespread dietary vitamin K insufficiency. Automated assays for two uncarboxylated MGP species are presently available: desphospho-uncarboxylated MGP (dp-ucMGP), a risk marker for cardiovascular disease and mortality, and total uncarboxylated MGP (t-ucMGP), a disease

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_14

marker for prevalent arterial calcification. The assay principles and the diagnostic utility of both markers are described in this chapter.

Keywords

Tissue calcification • Vascular stiffening • Mortality • Cardiovascular • Vitamin K • Menaquinone • Phylloquinone • ELISA • Conformation specific

Abbreviations

CAC	Coronary artery calcification
CVD	Cardiovascular disease
DMAE	Dimethyl acridinium ester
dp-ucMGP	Desphospho-uncarboxylated MGP
ELISA	Enzyme-linked immunosorbent assay
Gla	Gammacarboxyglutamate
HAMA	Human anti-mouse antigen
MGP	Matrix Gla protein
RLU	Relative light units
t-ucMGP	Total uncarboxylated MGP

Key Facts of Vascular Calcification

- Arterial calcification is a major risk factor for cardiovascular morbidity and mortality.
- Arterial calcification is an actively regulated process in which active MGP plays a key function.
- Atherosclerosis is an inflammatory process characterized by lipid accrual and plaque formation. Calcification is often seen at later stages of this process.
- Mönckeberg's sclerosis is the deposition of mineral in and around the elastic lamellae and vascular smooth muscle cells of the tunica media. It is not associated with inflammation and is characteristic for diabetes, chronic kidney disease, and aging.
- Mönckeberg's sclerosis leads to vascular hardening, loss of elasticity, increased pulse wave velocity, and hypertension.
- Vascular calcification is an actively regulated process with many similarities to bone formation. The vitamin K-dependent protein MGP plays a key role in the prevention of mineral deposition in the vasculature.

Definitions

Agatston score Score for the extent of calcification of the large arteries as quantified by electron beam computed tomography.

Gla domain The amino acid sequence in MGP in which four of its five Gla residues are clustered (residues 35–53).

iSYS The autoanalyzer produced by IDS Plc on which the MGP technology has been implemented.

Mönckeberg's sclerosis Calcification of the arterial medial layer.

Pser domain The amino acid sequence in MGP in which its three phosphoserine residues are clustered (residues 3–15).

Vascular calcification The deposition of calcium salts in the blood vessels.

Introduction

Whereas until the early 1990s vascular calcification was regarded as a passive process indicative for degeneration of the vessel wall, the deposition of calcium salts (mainly hydroxyapatite) in the arteries is now broadly accepted to be an actively regulated process (Sallam et al. 2013). Importantly, vascular calcification is known to be highly predictive for cardiovascular mortality (Willems et al. 2014). A key regulating protein is matrix Gla protein (MGP), the strongest inhibitor of soft tissue calcification presently known (Schurgers et al. 2008). It is synthesized locally (in the vessel wall by vascular smooth muscle cells and in cartilage by chondrocytes), but part of it is set free in the circulation where it is available for detection by ELISA-based technology (Cranenburg et al. 2010). This chapter describes (i) the principles of various assays available today, (ii) why it is important to quantify circulating MGP species in population-based and human intervention studies, and (iii) what is the potential importance of this biomarker for routine diagnostics of CVD patients and those at increased risk to develop CVD.

Matrix Gla Protein: Structure and Function

Gammacarboxyglutamate (Gla) is an unusual amino acid formed in the vitamin K-dependent posttranslational carboxylation of glutamate residues (Stafford 2005). Presently, 17 Gla proteins have been identified, and the presence of the Gla residues appeared to be essential for their function in all cases in which this function is known (Vermeer 2012). In its mature form, MGP is a small protein consisting of 84 amino acid residues, five of which are Gla (Price and Williamson 1985). Four Gla residues are clustered in what is generally referred to as the Gla domain. MGP also contains three serine residues which are potentially phosphorylated into the less common amino acid phosphoserine (Pser). Both Gla and Pser residues are negatively charged and form strong calcium-binding groups in MGP (Price et al. 1994). Remarkably, in

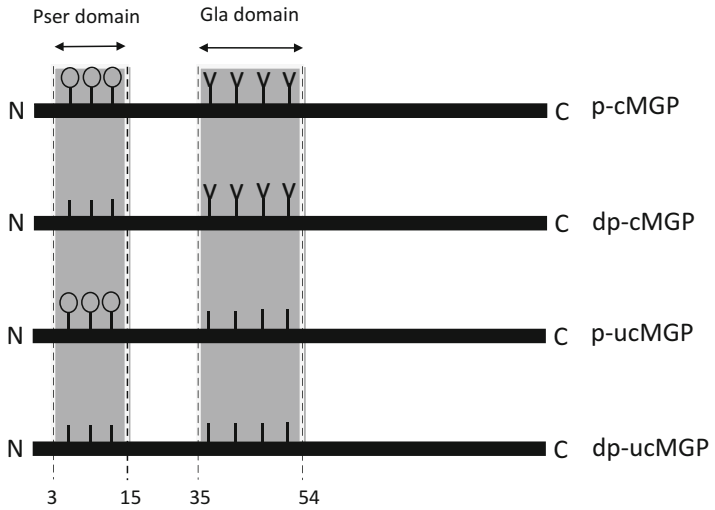

Fig. 1 Four conformations of MGP. Phosphoserine residues of MGP conformations are indicated by a globe and Gla residues by a V shape. Non-phosphorylated and non-carboxylated residues in the Pser and Gla domain are indicated by a single line. N and C represent the amino terminus and the carboxy terminus, respectively. The numbers 3–15 and 35–54 refer to the first and last amino acids in the Pser domain and in the Gla domain, respectively. In the dp-ucMGP assay, only MGP species completely devoid of the unusual amino acid residues Pser and Gla are detected. In the t-ucMGP assay, the sum of dp-ucMGP and p-ucMGP and degradation fragments containing the Gla domain are detected

the healthy adult population, both glutamate carboxylation and serine phosphorylation are exerted only in part of the MGP molecules, resulting in four different MGP conformations that are potentially formed and present (Fig. 1): MGP can be either phosphorylated or non-phosphorylated and either carboxylated or uncarboxylated. It is not sure whether also partially phosphorylated or carboxylated molecules exist. The completely unprocessed form (i.e., devoid of both Pser and Gla residues) has very little affinity toward calcium ions and no calcification inhibitory activity. This conformation is generally referred to as desphospho-uncarboxylated MGP (dp-ucMGP).

MGP is expressed in many tissues, but high levels of MGP protein are only found in cartilage and in the arterial vessel wall (Fraser and Price 1988; Shanahan et al. 1994). Its function was discovered in transgenic MGP-deficient mice, which were born normally but showed (i) cartilage calcification leading to maxillonasal hypoplasia and reduction of the length of the nasal bones, as well as growth retardation because of growth plate calcification and (ii) rapid calcification of all large arteries leading to fatal rupture of the aortas within 6–8 weeks after birth (Luo et al. 1997). In humans, MGP deficiency is known as the Keutel syndrome (Munroe et al. 1999), which was first described as a condition in which the cartilage of the ears, facial bones, and respiratory tract was heavily calcified and which also was associated with progressive artery calcification (Keutel et al. 1972). Because MGP is

a vitamin K-dependent protein, its activity can also be blocked by vitamin K antagonists such as warfarin. Price and coworkers developed an animal model allowing long-term treatment of rats with warfarin without the risk of fatal bleeding (Price et al. 1982). This model showed essentially the same phenotype as the $MGP^{-/-}$ mice: rapid calcification of the growth plates and arteries (Price et al. 1998; Howe and Webster 1992). Vitamin K antagonists (warfarin, acenocoumarol, and phenprocoumon) are broadly used to decrease thrombosis risk in patients, for instance, those with atrium fibrillation or artificial heart valves. Unfortunately, the adverse effects of these drugs discovered in experimental animal models are also seen in humans. Women taking vitamin K antagonists during the first 3 months of pregnancy are at very high risk of delivering a child with the fetal warfarin syndrome (chondrodysplasia punctata), which is characterized by excessive cartilage calcification leading to nasal dysplasia, stippled epiphyses, and skeletal deformations (Hall et al. 1980; Pauli et al. 1987). In adults, long-term oral anticoagulant treatment may lead to calcification of the large arteries and heart valves (Schurgers et al. 2004; Koos et al. 2005). Poor MGP carboxylation is the generally accepted explanation for these coumarin-induced ectopic calcifications (Kruger et al. 2013).

Physiological Importance of Calcification Inhibitors

Our body fluids and tissues are rich in calcium and phosphate ions, the concentration of which may exceed the solubility product of calcium phosphate (often referred to as the calcium x phosphate product). To prevent excessive soft tissue calcification, humans rely on a number of calcification inhibitors, the most important ones being fetuin-A (Ketteler et al. 2002) and MGP (Schurgers et al. 2008). Fetuin-A is a large protein ranging from 51 to 67 kD (depending on its carbohydrate content) and is abundantly synthesized in the liver (Jahnen-Dechent et al. 2011). In the circulation, it acts as a systemic inhibitor of soft tissue calcification. As was demonstrated by Price and coworkers, fetuin-A is perfectly capable of inhibiting calcification in body fluids and tissues, but it is too large to penetrate into the luminal side of collagen and elastin fibrils (Price et al. 2009). In the absence of small calcification inhibitors, these fibrils will therefore rapidly calcify. This principle is called mineralization by inhibitor exclusion. The vitamin K-dependent calcification inhibitors osteocalcin (in the bone) and MGP (in the cartilage and vessel wall), however, are sufficiently small to penetrate into the lumen of the fibrils and effectively inhibit mineralization at the inside. In order to optimally prevent vascular calcification, it is therefore critical for vascular health that MGP is fully active (i.e., fully carboxylated). Unfortunately, MGP is significantly under-carboxylated in the healthy adult population (Theuwissen et al. 2012b, 2014), and even more pronounced under-carboxylation was found in cardiovascular disease and diseases associated with high cardiovascular disease risk: diabetes mellitus and chronic kidney disease (Liabeuf et al. 2014; Dalmeijer et al. 2013c; Schurgers et al. 2010). This is consistent with the fact that poor dietary vitamin K intake was found to be associated with increased cardiovascular morbidity and (Geleijnse et al. 2004; Gast et al. 2009) and also with the well-

documented under-carboxylation of another extrahepatic Gla protein, osteocalcin (Liabeuf et al. 2014; Booth et al. 2004; Szulc et al. 1993). These observations formed the basis for the concept that the plasma concentration of circulating uncarboxylated MGP fractions may be markers in cardiovascular disease and can be used for estimating cardiovascular disease risk and mortality. Conformation-specific antibodies were prepared at VitaK to design conformation-specific tests, and their diagnostic utility was demonstrated and patented (Vermeer and Braam 2001; Schurgers et al. 2005). Most of the data in the literature were generated with homemade microtiter plate assays, but after the patents were transferred to Immunodiagnostics Plc (IDS, Boldon, UK), the R&D Group VitaK and IDS have jointly worked out the automation of the two most successful MGP-based assays, the marketing of which is expected within shortly.

Two Tests: dp-ucMGP and t-ucMGP

Desphospho-uncarboxylated MGP (dp-ucMGP) is the fraction of MGP that enters the circulation devoid of posttranslational modifications. Because it lacks calcium-binding groups, its affinity for calcium ions and hydroxyapatite is low, and it has no calcification inhibitory activity. A sandwich ELISA has been developed in which monoclonal antibodies against the non-phosphorylated Pser domain (amino acid residues 3–15 in human MGP) were used as capture antibodies and monoclonal antibodies against part of the non-carboxylated Gla domain (amino acid residues 35–49) as detection antibodies (Cranenburg et al. 2010). The normal range for circulating dp-ucMGP is 200–600 pM; high values reflect poor vascular vitamin K status, and low values reflect high vascular vitamin K status. Patients using vitamin K antagonists (oral anticoagulants) have extremely high dp-ucMGP levels, whereas those on vitamin K supplements may have levels below 50 pM. The product detected with this assay was identified by immunoprecipitation followed by western blot analysis and mass spectronomy analysis (Cranenburg et al. 2008). In Fig. 2, the same technique was used using the anti-ucMGP antibodies for immunoprecipitation and anti-dpMGP and ucMGP antibodies for identifying MGP species on the membrane, visualized by fluorescence detection using a fluorescently labeled rabbit-anti-mouse antibody. The fact that a single band of approximately 11 kD was obtained demonstrates that only intact dp-ucMGP is identified with the dp-ucMGP ELISA. Although the microtiter plate assay is still available at VitaK's Center for Vascular Diagnostics, the test has been transferred to the IDS-iSYS autoanalyzer and is commercialized as the Ina*K*tif MGP assay. The principles of this assay are explained in Fig. 3. A comparison between the microtiter plate ELISA and the Ina*K*tif MGP assay showed a strikingly good correlation ($r^2 = 0.95$, $p < 0.0001$, see Fig. 4), which means that data obtained with both assays are comparable. Both assays are robust and can be used in samples that have been stored at −80 °C for 10 years or longer, but a disadvantage is that their use is limited to plasma (EDTA or citrate); in serum, most of the signal is lost. In both assays, a synthetic standard is used as a reference; this standard is composed of the non-phosphorylated 3–15 peptide and the

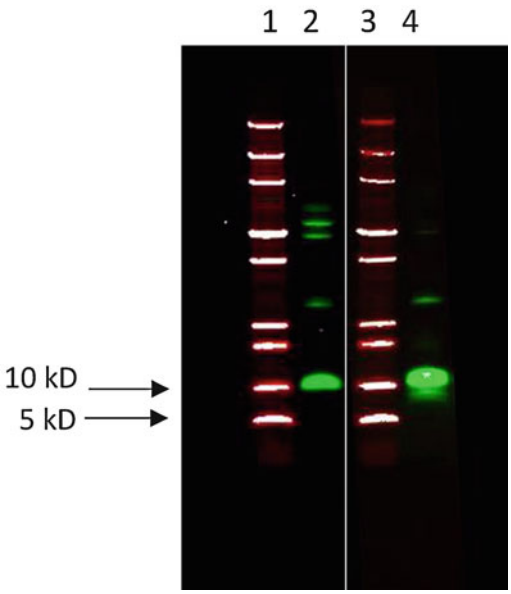

Fig. 2 Western blot analysis of MGP species. For immunoprecipitation and western blot analysis of MGP species, immobilized anti-ucMGP was used to extract plasma, whereafter the bound material was eluted with elution buffer (Pierce Direct IP kit), separated by SDS PAGE and transferred to a nitrocellulose membrane. Lanes *1* and *3*: molecular weight markers; *arrows* indicate MW of 5 and 10 kD. Lane *2*: dp-ucMGP; Anti-dpMGP was used as a detection antibody, and staining was accomplished with a fluorescently labeled secondary antibody (donkey anti-mouse total IgG). The Odyssey Infrared Imaging System (Li-Cor) was used for the detection of the fluorescently labeled antibody. Lane *4*: the same procedure as for lane *2* except that anti-ucMGP was used for detection. In lane *2* a single band was observed at 11 kD, which is consistent with full-length dp-ucMGP. In lane *4* also some smaller material was observed, indicative for ucMGP degradation products

uncarboxylated 35–54 peptide, connected by a linker molecule. Using the phosphorylated 3–15 peptide, no cross-reactivity was found even at very high concentrations; at equimolar concentrations of carboxylated 35–54 peptide and synthetic standard peptide, 14 % of the signal was lost, suggestive for minor cross-reactivity with cMGP. The intra-assay variations of the homemade and the Ina*K*tif MGP assay were 6 % and 5 %, respectively, and the inter-assay variations were 10 % and 7 %. Because of the much better linearity upon dilution, the automated assay has a much broader measuring range than the microtiter plate ELISA.

Total uncarboxylated MGP (t-ucMGP) is the fraction of MGP that enters the circulation in the uncarboxylated state, independent of its phosphorylation. A competitive ELISA has been developed in which monoclonal antibodies against the non-carboxylated Gla domain of human MGP were used as capture antibodies and a biotinylated synthetic peptide (containing the entire Gla domain) as a tracer (Cranenburg et al. 2010). The normal range for circulating t-ucMGP was found to be 2000–10,000 nM, which is roughly 10,000-fold higher than that of dp-ucMGP. In

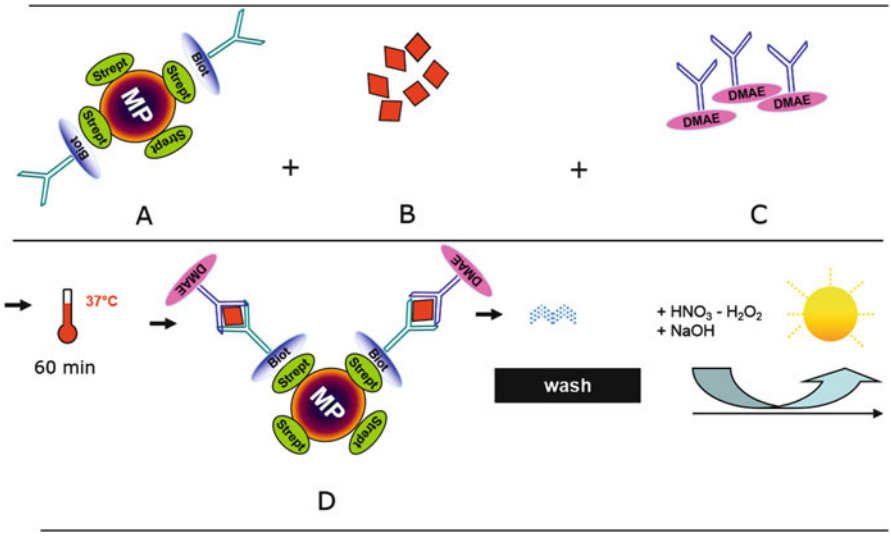

Fig. 3 Principle of the IDS-iSYS Ina*K*tif MGP assay. For the IDS-iSYS Ina*K*tif MGP assay, streptavidin-coated magnetic particles (MP) are linked to biotinylated monoclonal conformation-specific antibodies against dp-MGP (**a**) and incubated with a plasma sample containing native dp-ucMGP (**b**) and soluble DMAE-conjugated monoclonal conformation-specific antibodies against uncarboxylated MGP (**c**). Incubation is for 60 min at 37 °C. The resulting sandwich complex (**d**) is washed and incubated with trigger reagents (HNO_3 + H_2O_2); the light emitted by the acridinium label is measured and is expressed in relative light units (RLU). The signal produced is directly proportional to the dp-ucMGP concentration in the sample. DMAE stands for dimethyl acridinium ester (Figure Courtesy by M. Bougoussa and D. Ziant (IDS Plc, Liege, Belgium))

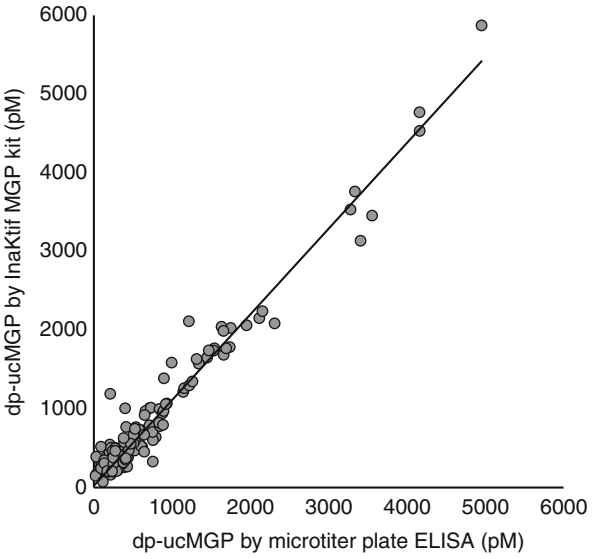

Fig. 4 Correlation between dp-ucMGP assays. Correlation between microtiter plate dp-ucMGP ELISA and Ina*K*tif MGP assay. One hundred EDTA plasma samples from 50 untreated subjects, 25 low-dose vitamin K-treated subjects, and 25 subjects on long-term oral anticoagulant treatment were measured in the homemade ELISA and in the IDS-iSYS autoanalyzer. The mathematical equation for correlation was $y = 1.0882$ x, with an R^2 of 0.9554

part this can be explained by the higher affinity of the antibodies used for the synthetic peptide (against which they were raised), and theoretically the assay will also detect fragmented MGP containing the uncarboxylated Gla domain. The product detected with this assay, however, consisted almost entirely of 11 kD MGP. This was demonstrated by immunoprecipitation using anti-ucMGP antibodies, followed by western blot analysis also using anti-ucMGP antibodies for detection, and fluorescent staining as described before (see Fig. 2). The strong band at 11 kD was accompanied by a minor shade of low molecular weight material, indicating that by far the majority of the ucMGP species found in the circulation is intact and may be in a phosphorylated conformation. This would endow the molecule with high-affinity sites for calcium and might explain the fact that circulating t-ucMGP is inversely associated with the extent of vascular calcification (see also below). A fact still poorly understood is that circulating t-ucMGP is not affected by vitamin K intake or oral anticoagulants. One explanation may be that the majority of the t-ucMGP found in plasma originates from decarboxylation of preexisting carboxylated MGP, but this hypothesis needs to be verified. Although the microtiter plate assay for t-ucMGP is still available at VitaK's Center for Vascular Diagnostics, the test has been transferred to the IDS-iSYS autoanalyzer which is now commercially available. The principles of this assay are explained in Fig. 5. A comparison between the microtiter plate ELISA and the automated assay showed a good correlation ($r^2 = 0.76, p < 0.0001$, see Fig. 6); the reason why the correlation is less than for the dp-ucMGP assays is that the linearity of the automated assay in the higher ranges is much better than that of the homemade assay. Both assays are robust and can be used in samples that have been stored at −80 °C for 10 years or longer, and the advantage of the t-ucMGP over the dp-ucMGP is that it can be used both in plasma and in serum. Both t-ucMGP assays use the same synthetic standard that is also used as a reference in the dp-ucMGP tests: the non-phosphorylated 3–15 peptide and the uncarboxylated 35–54 peptide, connected by a linker molecule. As with the dp-ucMGP tests, 14 % of the signal was lost at equimolar concentrations of tracer and carboxylated 35–54 peptide, suggestive for minor cross-reactivity with cMGP. The intra-assay variations of the homemade and the automated t-ucMGP assay were 9 % and 6 %, respectively, and the inter-assay variations were 12 % and 7 %.

Dp-ucMGP: A Marker for CVD Risk and Mortality

Dp-ucMGP is a marker for vascular vitamin K status, but it remains unaffected by short-term variations in dietary vitamin K intake. Only after 2 weeks of increased daily vitamin K intake, the circulating dp-ucMGP levels start to decline to reach a new steady state only after 8–12 weeks of high vitamin K intake (Theuwissen et al. 2012a; Liabeuf et al. 2014). Remarkably, in population-based studies, the assay predicted not only the risk for cardiovascular morbidity and mortality but also the overall mortality. Presently, 14 large cohort studies have been published (see Table 1) and three others are in press. All studies showed a strong association between plasma dp-ucMGP and cardiovascular calcification/disease risk or mortality, with higher dp-ucMGP values correlating with increased CVD risk. The one

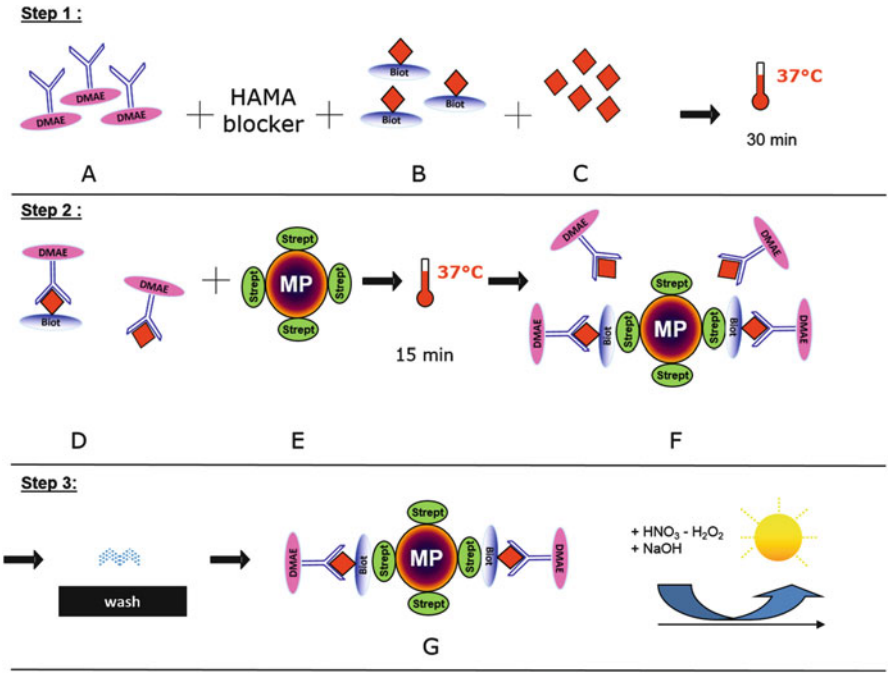

Fig. 5 Principle of the IDS-iSYS t-ucMGP assay. Steps showing the principle of the IDS-iSYS t-ucMGP assay. *Step 1*: A limiting amount of monoclonal anti-ucMGP antibodies linked with DMAE are incubated for 30 min at 37 °C with HAMA-blocker solution and a mixture of biotinylated tracer (**b**) and plasma or serum containing native MGP (**c**). *Step 2*: The antibody-antigen complexes thus formed are incubated at 37 °C for 15 min with magnetic particles (*MP*) coated with streptavidin to give captured and free complexes. *Step 3*: Unbound antibody-antigen complexes are removed in a washing step, and the remaining insolubilized tracer-antibody complexes (**g**) are quantified by adding with trigger reagents (HNO_3 + H_2O_2); the light emitted by the acridinium label is measured and is expressed in relative light units (RLU). The signal produced is inversely proportional to the t-ucMGP concentration in the sample. DMAE stands for dimethyl acridinium ester (Figure Courtesy by M. Bougoussa and D. Ziant (IDS Plc, Liege. Belgium))

exception (no association) was a study in which the potential association with coronary heart disease was tested (Dalmeijer et al. 2014). However, all dp-ucMGP values in this study were extremely low (around the lower detection limit of the assay), which was probably caused by suboptimal sample storage resulting in lack of power. In one population-based study among 2500 subjects with a 12-year follow-up period, it was found that increased dp-ucMGP levels explain 22 % of all cardiovascular mortality (Liu et al. 2015). The assay turned out to have a very high predictive value for cardiovascular outcomes in chronic kidney patients (Cranenburg et al. 2010, 2012; Boxma et al. 2012). These patients are characterized by a very high risk for vascular calcification, which at least in part may be caused by the diet they have to follow (Schlieper et al. 2008). Typically, these diets are low in vitamin K (Boxma et al. 2012) and rich in phosphate resulting in a high calcium x phosphate product in their blood plasma (Schlieper et al. 2009), combined with poor MGP

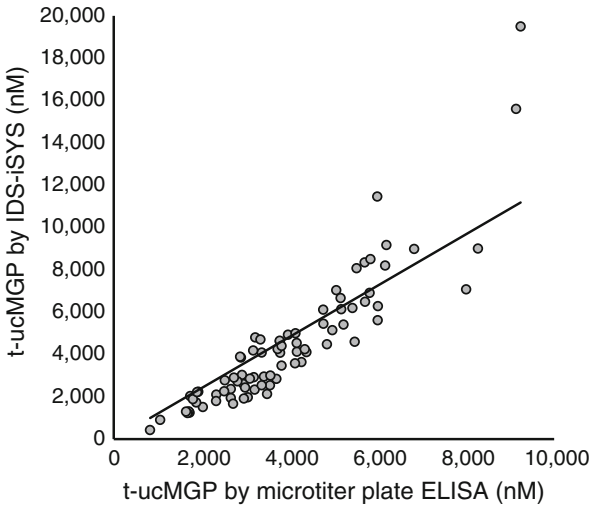

Fig. 6 Correlation between t-ucMGP assays. Correlation between microtiter plate t-ucMGP ELISA and IDS-iSYS t-ucMGP assay. One hundred EDTA plasma samples stratified for a broad range of t-ucMGP values were measured in the homemade ELISA and in the IDS-iSYS autoanalyzer. The mathematical equation for correlation was $y = 1.2096$ x, with an R^2 of 0.7555

Table 1 Association of circulating dp-ucMGP with cardiovascular disease risk and mortality risk

Reference	Study population	Outcomes
van den Heuvel et al. 2014	Healthy subjects	Association with CVD mortality risk
Liabeuf et al. 2014	Type-2 diabetics	Association with arterial calcification risk
Mayer et al. 2014	Vascular disease	Predicts mortality risk
Dalmeijer et al. 2014	Coronary heart disease	No association found[a]
Keyzer et al. 2015	Renal transplantation	Predicts allograft failure and mortality risk
Liu et al. 2015	Population based	Associated with risk for cardiovascular mortality
Caluwe et al. 2014	Hemodialysis	Very high levels, decreased with vitamin K2
Dalmeijer et al. 2013c	Healthy women	Predicts coronary artery calcification risk
Dalmeijer et al. 2013a	Healthy women	Associated with vascular calcification risk
Dalmeijer et al. 2013b	Type-2 diabetics	Associated with risk for cardiovascular events
Westenfeld et al. 2012	Hemodialysis	Very high levels, decreased with vitamin K2
Ueland et al. 2011	Chronic heart failure	Associated with disease severity
Schurgers et al. 2010	Chronic kidney disease	Surrogate marker for vascular calcification risk
Cranenburg et al. 2010	Aortic valve disease	Strongly elevated plasma levels
Cranenburg et al. 2010	End-stage renal disease	Strongly elevated plasma levels
Ueland et al. 2010	Aortic stenosis	Associated with development of heart failure

All studies were population-based studies with a long-term (5–15 years) follow-up, in which plasma dp-ucMGP was assessed only at baseline

[a]Extremely low dp-ucMGP levels (probably because of suboptimal sample storage), resulting in loss of power

carboxylation and thus with low vascular calcification inhibitory activity. This has led to the hypothesis that increased vitamin K intake might decrease vascular calcification in these patients (Krueger et al. 2009). Remarkably, also in apparently healthy kidney transplant recipients, the dp-ucMGP assay was a strong predictor for allograft failure and life expectancy (Keyzer et al. 2015), a fact that may be related to the sustained dietary habits of low vitamin K and high phosphate intake. It may be wise, therefore, to continue monitoring dp-ucMGP in allograft recipients and on guidance of their vitamin K status encourage them to increase vitamin K intake (green vegetables, curds, and cheeses). In human intervention studies, it was found that the high dp-ucMGP levels in hemodialysis patients could be decreased by vitamin K supplements (Westenfeld et al. 2012; Caluwe et al. 2014), and several clinical intervention studies with high doses of vitamin K are in progress among this patient group. In two 3-year intervention studies among healthy subjects, increased vitamin K intake resulted in beneficial vascular outcomes (no age-related loss of elasticity (Braam et al. 2004) and substantial decrease of pulse wave velocity (Knapen et al. 2015). All data presently available indicate that even slightly elevated dp-ucMGP values are a strong risk factor for cardiovascular disease, and the assay is particularly suited to monitor subjects who wish to decrease this risk factor by increasing their vitamin K intake. Moreover, dp-ucMGP is a strong potential confounder in all studies with a cardiovascular outcome and should be assessed for proper statistical evaluation of the outcomes obtained in those studies.

T-ucMGP: A Marker for Prevalent Vascular Calcification

Circulating t-ucMGP was quantified in at least nine published studies (summarized in Table 2) and in three studies in press. It was invariably found that t-ucMGP is inversely associated with the extent of vascular calcification or is very low in patients known to be at high risk for ectopic calcification (diabetes, hemodialysis, calciphylaxis). This would be consistent with the presence of one or more calcium-binding groups in the majority of the t-ucMGP species (see above) detected with this assay and also with the fact that by immunohistochemical techniques high concentrations of ucMGP were found in close association with calcium salt deposits in the arterial tunica media (e.g., in Mönckeberg's sclerosis, (Schurgers et al. 2005)). In one case (Parker et al. 2010a), also an inverse association was found with all-cause mortality, which is to be expected because vascular calcification is predictive for cardiovascular events and mortality. The predictive power for future events of the dp-ucMGP assay is better than that of the t-ucMGP assay, however. The potential application of the t-ucMGP assay seems mainly to be its use as a disease marker for prevalent arterial calcification. Although the results published until now are promising, there is a need for some large studies in which calcification of the large arteries is quantified by electron beam computed tomography (Agatston score) and expressed as a function of circulating t-ucMGP. Whether t-ucMGP can be used as a marker to monitor the progress of calcification or the effect of treatment is presently unknown.

Table 2 Association of circulating t-ucMGP with cardiovascular disease and calcification

Reference	Study population	Outcomes
Dalmeijer et al. 2013c	Healthy women	Inverse association with CAC
Dalmeijer et al. 2013c	Postmenopausal women	Inverse association with CAC
Parker et al. 2010a	Coronary artery disease	Inverse association with mortality risk
Parker et al. 2010b	Diabetes mellitus	Association with mitral annular calcification
Cranenburg et al. 2010	Various populations	Very low in ESRD and aortic valve disease
Parker et al. 2009	Stable CVD	Association with glomerular filtration rate
Parker et al. 2009	Hemodialysis patients	Inverse association with CAC
Cranenburg et al. 2008	Various populations	Lower t-ucMGP in subjects known to be at risk for arterial calcification
Schurgers et al. 2005	Percutaneous coronary intervention	Significantly lower than in control population

Most studies were cross-sectional with t-ucMGP measurement at the same time as disease assessment (calcification score, etc.)

Potential Application of ucMGP Tests in Research and in the Clinical Setting

Plasma dp-ucMGP is a biomarker of which the measurement is highly recommendable in human intervention trials with cardiovascular endpoints; at baseline, it should be used for stratification of study groups, whereas during the study, it can be used to monitor the effect of treatment. A critical question here is whether dp-ucMGP should be normalized by supplemental vitamin K intake in case effects of other medications are tested. Moreover, dp-ucMGP is an important factor in multivariate analysis of the outcomes of such studies. Also in population-based studies investigating cardiovascular disease, dp-ucMGP should be included as a potential confounder. Since the assay basically measures vitamin K status, it may also become important for other conditions reported to be associated with poor vitamin K status including osteoporosis (Hart et al. 1984; Szulc et al. 1993; Booth et al. 2003), osteoarthritis (Misra et al. 2013; Oka et al. 2009; Neogi et al. 2006), diabetes mellitus (Liabeuf et al. 2014; Yoshida et al. 2008; Dalmeijer et al. 2013c), or cognitive function (Ferland 2013; Presse et al. 2013), but this is presently unclear. Although the dp-ucMGP assay has not yet been introduced as a routine marker for risk assessment in individual cardiovascular patients, its potential utility is large. Population-based studies have unequivocally demonstrated that elevated dp-ucMGP levels form a high and independent risk factor for cardiovascular disease, and the fact that increased vitamin K intake quickly improves MGP carboxylation and thus eliminates this risk factor

demonstrates its potential benefit for public health, notably in the prevention of vascular calcification. Moreover, two independent intervention trials demonstrated the positive effect of vitamin K supplements on vascular elasticity and pulse wave velocity. Also in animal models, calcification was prevented (Spronk et al. 2003) and even reversed (Schurgers et al. 2007) by high vitamin K intake. Therefore, measuring dp-ucMGP may save many lives by early detection of inadequacy of vitamin K and supplementing these subjects with vitamin K.

Circulating t-ucMGP is a promising marker for rapid screening of subjects who may be in need of more invasive vascular diagnostics. Preliminary data show that low t-ucMGP levels are indicative for prevalent vascular calcification, but in some aspects, the assay is still poorly understood. The fact, for instance, that high doses of vitamin K or vitamin K antagonists (warfarin) have no effect on the circulating t-ucMGP level raises questions about the nature and the origin of the detected antigen. Also the mechanism underlying the observed inverse association between t-ucMGP and the extent of arterial calcification remains to be explained. Therefore, the t-ucMGP assay is not yet ready for routine diagnostics but forms a valuable tool for researchers who want to stratify study groups for prevalent calcification or who want to eliminate vascular calcification as a confounding factor in their data analysis. If this assay will reach the stage of routine diagnostics, however, it may prove to be a rapid and cost-effective method to help discriminate between patients in need for more elaborate (and expensive) vascular diagnostics and those with relatively fewer artery calcifications.

Summary Points

- Dp-ucMGP has been extensively validated in patients and in healthy subjects. It measures vitamin K insufficiency, and high circulating levels predict cardiovascular disease risk and mortality.
- Most importantly, circulating dp-ucMGP is rapidly brought down to normal or subnormal levels by vitamin K2 supplements with dosages that are in the nutritional range.
- Decreasing dp-ucMGP by increased K intake was found to be associated with improving vascular condition, which provides a simple tool to help prevent CVD and even support therapeutic interventions.
- Even in case of unchanged vitamin K intake, dp-ucMGP may be a tool for follow-up of treatment (diet or medication) in CVD patients.
- T-ucMGP is an attractive second assay but needs further validation. Notably its association with extent of arterial calcification (Agatston score) needs to be confirmed in independent cohorts.

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MicroRNA-133: Biomarker and Mediator of Cardiovascular Diseases

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_28

Abstract

If we consider the molecular size of major nucleic acids and the time elapsed since we knew about their existence, microRNAs (miRs) comparatively appear as recently discovered and structurally small pieces of genetic material, but we should make no mistake to underestimate these modest but powerful molecules. Regulating no less than half of the transcriptome, their influence is a key for the fine-tuning of almost any biological process. miRs are considered elements that add precision and robustness to a myriad of physiological and pathological processes and commonly function as components of cellular networks by buffering extremes in gene expression. Their small size limits the side effects of these molecules and eases a pervasive behavior that explains their presence in different body fluids but also opens a complete new field of clinical opportunities for diagnostic, prognostic, or risk stratification applications which benefit from the possibility of bedside measurement.

From a purely investigational perspective, miRs constitute also a very useful tool. Their pleiotropic repressing effect on various, often functionally related, target mRNAs gives us clues on the molecular mechanisms underlying many pathological phenomena. On the other hand, since mRNA complementarity is dictated by a relatively short sequence (seed region) of nucleotides in the miR, computer-based prediction of miR targets is readily available. Additionally, "the control on the controllers" is tempting with the aim of modifying favorably the natural history of miR-related diseases. Several techniques to block or overexpress miRs have been launched, and, even though there are significant difficulties and drawbacks in their administration, some of them are starting to be used in human therapeutics.

miR-133 is muscle specific and one of the most abundant miRs in the heart. It plays major roles in the developing heart on cell differentiation into muscle tissue and also in later stages of cardiac morphogenesis. It is included among the handful of molecules able to cooperate for the experimental reprogrammation of fibroblasts into cardiac-like myocytes. miR-133 participates in the molecular pathology of myocardial hypertrophy, be it primary or secondary, in the transition and evolution of cardiac failure, in myocardial fibrosis and in cardiac cell apoptosis. In stable coronary artery disease and, particularly, in the acute coronary events, miR-133 levels decrease in the myocardium and increase in the circulation proportionally to the extent of the infarcted area. The value of circulating levels of miR-133 as a diagnostic and prognostic biomarker in these patients during the acute ischemic episode and after primary revascularization has already been established. Finally, research studies undertaken during the last 5 years show that dysregulation of miR-133 contributes to the pathological vascular remodeling underlying essential hypertension, vascular calcification, atherosclerosis, and aneurysmal disease.

At this time, it could be said that miRs are ready for prime time for diagnostic and prognostic purposes in the clinical arena, but more is coming on miR molecular manipulation with miR mimics and anti-miRs and even more with targeting of miR-related pathways. Meanwhile, other future uses of miRs in the

fields of regenerative medicine and tissue engineering await for further refinement before they can be clinically applicable.

Keywords

Biomarkers • MicroRNAs • miR-133 • Cardiogenesis • Cardiac remodeling • Left ventricular hypertrophy • Myocardial fibrosis • Arterial aneurysm • Coronary arterial disease • Vascular smooth muscle

Abbreviations

AAA	Abdominal aortic aneurysm
ACS	Acute coronary syndrome
AMI	Acute myocardial infarction
BAV	Bicuspid aortic valve
ceRNA	Competing endogenous RNA
CHD	Coronary heart disease
CTGF	Connective tissue growth factor
cTn	Cardiac troponin
ICA	Intracranial aneurysm
lnc-RNA	Long noncoding RNA
LV	Left ventricle
LVAD	Left ventricular assist device
MI	Myocardial infarction
miR	MicroRNA
MMP	Matrix metalloproteinase
MRE	miR recognition element
NSTEMI	Non ST-elevation myocardial infarction
qRT-PCR	Real-time polymerase chain reaction
ROC	Receiver operating characteristic
STEMI	ST-elevation myocardial infarction
TAA	Thoracic aortic aneurysm
TAC	Transverse aortic constriction
TAD	Thoracic aortic dissection
TAV	Tricuspid aortic valve
TGF-β	Transforming growth factor-β
UA	Unstable angina
UTR	Untranslated region
VSMC	Vascular smooth muscle cell

Key Facts of MicroRNAs in Cardiovascular Disease

- The World Health Organization estimates that in 2015 there will be about 20 million deaths due to cardiovascular diseases, accounting for 30 % of the global death toll. Cardiovascular disease will continue to dominate mortality trends in the near future.

- Early detection of disorders before severe cardiovascular damage takes place may benefit from the identification of new tissue-specific biomarkers that can improve the sensitivity and accuracy of the diagnostic process.
- Aside from their value for risk assessment, early diagnosis, and prognosis, biomarkers may also act as mediators of disease, what underscores their interest in the elucidation of the pathogenesis of cardiovascular disease.
- miRs are powerful regulators of many, if not all, biological processes in the cell, and their dysregulation plays important roles in cardiovascular diseases.
- More than 2,500 miRs have been identified in humans. One miR can modify the translation to protein of up to hundreds of different mRNAs, and a single mRNA can be targeted by several miRs.
- Manipulation of miR expression has emerged as a valuable tool for therapeutic intervention, and, despite their recent discovery, several miR candidates have already progressed into clinical development.
- miRs are released by cells, remain in the circulation in a stable cell-free form, and can be detected by sensitive, specific, and minimally invasive methods. These characteristics make miRs potential bedside biomarkers for cardiovascular diseases.

Definitions

Anti-miRs Synthetic single-stranded antisense oligonucleotides that hybridize by base pairing to target miRs and block their function.

Cardiac remodeling Genome expression resulting in molecular, cellular, and interstitial changes and manifested clinically as changes in size, shape, and function of the heart originated from cardiac load or injury.

MicroRNAs (miRs) Short non-coding, single-stranded RNAs involved in the posttranscriptional control of gene expression. miR binding to its mRNA targets results in the inhibition of translation to proteins.

miR-133 Muscle-specific miR that is expressed by skeletal and cardiac striated myocytes and by smooth muscle cells.

miR cluster miRs transcribed as a single unit from the same primary transcript that are regulated in a similar way.

miR family Group of miRs that have identical seed regions and share targets and functions. The occurrence of miRs belonging to distinct "seed" families within the same cluster is common.

miR recognition element (MRE) mRNA sequence in the 3′-UTR end that binds a specific miR usually by partial or complete base pairing.

miR sponges Are synthetic RNA molecules harboring complementary binding sites to the seed sequences of a given miR or a miR family.

Myomirs Group of miRs that are highly expressed in muscular tissue.

Seed region Sequence of 6–8 nucleotides at the 5′ end of miRs which binds by base pairing with complementary sequences of target mRNAs.

Transverse aortic constriction Experimental model of LV pressure overload induced by surgical banding of the mid aortic arch.

Introduction

MicroRNAs (miRs) are short (~22 nucleotide long) noncoding, evolutionary conserved, single-stranded RNAs involved in the posttranscriptional control of gene expression. miRs bind to mRNA transcripts that have complementary target sequences to produce mRNA silencing, either through the inhibition of mRNA translation or, less often, by the induction of mRNA degradation (Ha and Kim 2014). miRs were first described in the nematode *Caenorhabditis elegans* as regulatory factors that control the expression of genes involved in larval development (Lee et al. 1993). Since then, more than 2,500 mature miRs have been identified in humans, and each of them can modify the translation of up to hundreds of different mRNAs. The role of miRs as powerful regulators of many, if not all, biological processes in the cell has been widely studied, and their dysregulation plays important roles in numerous human diseases. Identification and validation of miR targets are steps of fundamental importance for the comprehensive understanding of miR roles in physiological and pathological conditions and, therefore, of their possible therapeutic applications (Dangwal and Thum 2014). miRs can be released by cells and remain in the circulation in a remarkably stable cell-free form. Detection of miRs can be sensitive, specific, and minimally invasive. Such characteristics warranted a general interest on circulating miRs as potential measurable bedside biomarkers for detecting a wide range of diseases, including those affecting the cardiovascular system, and for monitoring disease conditions and efficacy of therapeutic regimens (Creemers et al. 2012).

miR-133a is one of the most abundant miRs in the heart. The combination of experimental models of human pathologies with tools that modulate miR-133 activity has provided important insights on the role played by this miR and their targets in very prevalent cardiovascular pathologies (van Rooij and Kauppinen 2014). It contributes to cell differentiation into muscle tissue during development and in later stages of cardiac morphogenesis. It is included among the handful of molecules able to cooperate for the experimental reprogramming of fibroblasts into cardiac-like myocytes. miR-133 participates in the molecular pathology of myocardial hypertrophy, be it primary or secondary, in the transition to and evolution of cardiac failure, in myocardial fibrosis and in cardiac cell apoptosis. In stable coronary artery disease and, particularly, in the acute coronary events, miR-133 levels

decrease in the myocardium and increase in the circulation proportionally to the extent of the infarcted area. The value of circulating levels of miR-133 as a diagnostic and prognostic biomarker in these patients during the acute ischemic episode and after primary revascularization has already been established. Finally, research studies undertaken during the last 5 years show that dysregulation of miR-133 contributes to the pathological vascular remodeling underlying essential hypertension, vascular calcification, atherosclerosis, and aneurysmal disease.

MicroRNA Biogenesis, Structure, and Biology

The biogenesis of a functional miR (Fig. 1) (Ha and Kim 2014) starts in the nucleus with the transcription of primary transcripts (pri-miRs) by RNA polymerase II from intronic, exonic, intergenic, or polycistronic loci. The pri-miR has a stem-loop structure that incorporates one or more miR-encoding precursors. pri-miR is processed in the nucleus by a protein complex formed by the RNAse III DROSHA and DGCR8 (DiGeorge syndrome chromosomal region 8) to a ~70 nucleotide hairpin-shaped precursor miR (pre-miR), which contains the mature miR in either the 5′ or 3′ arm. Pre-miRs are transported to the cytoplasm, where the RNase III DICER and its cofactor TRBP (the human immunodeficiency virus transactivating response RNA-binding protein) cleave the pre-miR hairpin and separate the loop from the double-stranded stem, forming miR duplexes comprised by the passenger and the guide strands. DICER and the miR duplex integrate into a complex with argonaute proteins, which loads the guide strand to the miR-induced silencing complex (RISC). Usually, the guide strand of the miR duplex is the mature and active miR form that guides RISC to target mRNAs, while the passenger strand is degraded. However, for some miRs, the passenger strand can be also active. The most critical determinant for miR targeting of mRNAs is the "seed region," constituted by six to eight nucleotides at the 5′ end of miRs. Nucleotides at the seed region form Watson-Crick pairs with complementary sequences, called miR recognition elements (MREs), in the 3′-UTR end of the mRNA, producing either repression of translation or destabilization of the target mRNA (Ha and Kim 2014). Recent studies have shown that the functional consequences of the interaction between miR's seed regions and mRNAs are not univocal. The so-called competing endogenous RNAs (ceRNAs) are RNA transcripts [mRNAs, transcribed pseudogenes, and long noncoding RNAs (lnc-RNAs)] that, by sharing MREs, compete for binding to miRs and then regulate each other's expression. The cross talk between ceRNA-miRNA-mRNA maintains the functional balance of gene networks in the cell (Salmena et al. 2011).

MicroRNA Targets

The reliable prediction of potential miR targets is possible with computational methods. Silico predictions often use algorithms which account for interactions between the 3′-UTR sequences of mRNAs and the seed regions of miRs, the

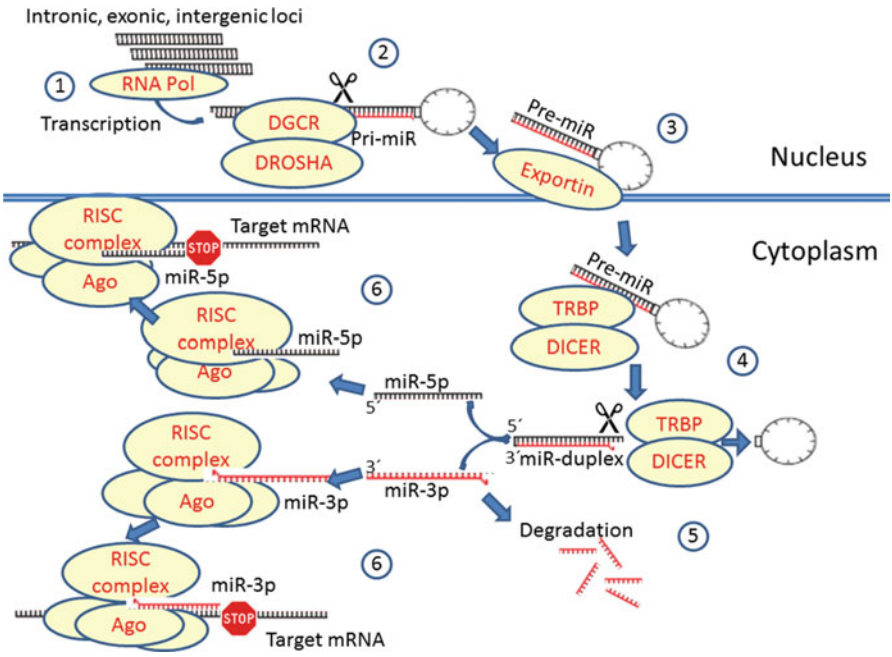

Fig. 1 Biogenesis of a functional miR. The biogenesis of a functional miR starts in the nucleus with the transcription of primary transcripts (*pri-miRs*) by RNA polymerase II from intronic, exonic, intergenic, or polycistronic loci (*1*). The pri-miR is processed in the nucleus by a complex formed by the RNAse III DROSHA and DGCR8 (DiGeorge syndrome chromosomal region 8) to a hairpin-shaped precursor miR (*pre-miR*), which contains the mature miR in either the 5′ or 3′ arm (*2*). Pre-miRs are transported by exportin to the cytoplasm (*3*), where the RNase III DICER and its cofactor TRBP (the human immunodeficiency virus transactivating response RNA-binding protein) cleave the pre-miR hairpin, forming miR duplexes of passenger and guide strands (*4*). DICER and the miR duplex integrate into a complex with argonaute proteins, which loads the guide strand to the miR-induced silencing complex (*RISC*). Usually, the passenger strand is degraded (*5*) and the guide strand is the active form that guides RISC to target mRNAs (*6*). For some miRs, the passenger strand can be also active (*6*). The interaction miR-mRNA represses the translation of targeted mRNAs to proteins

presence of multiple target sites, the thermodynamic stability of miR-mRNA hybrid, and the phylogenetic conservation. Today, many web-accessible resources organize all kinds of miR-related data and provide information regarding predicted and experimentally verified targets, biological- and disease process-related miRs, tissue expression patterns, regulatory networks and signaling pathways, etc. Representative examples are the following: HMDD; miRanda, miRBase; miR2Disease; miRSearch; miRecords; PicTar; PITA; TarBase; Targetscan; etc.

It is known that 3′-UTR sequences with perfect complementarity with the miR are not necessarily functional, while mRNA sites with imperfect pairing can be very good miR targets; therefore, bioinformatic predictions are prone to false positives. However, there are multiple exceptions regarding the requirement for miR-binding sites to be located in the 3′ UTR. Thus, all miR targets have to be validated in vitro

and in vivo since prediction programs only propose plausible candidates for validation.

Besides their intracellular functions, recent studies demonstrate that miRs can be released by cells and remain in the circulation in a remarkably stable cell-free form. miRs have been found in various body fluids, including serum, plasma, saliva, tears, urine, amniotic fluid, colostrum, breast milk, bronchial lavage, cerebrospinal fluid, peritoneal fluid, pleural fluid, and seminal fluid. miRs are easily detected and quantified in body fluids by microarray assays, next-generation sequencing, northern blot, and real-time polymerase chain reaction (qRT-PCR). Detection of miRs can be sensitive, specific, and minimally invasive. Such characteristics warranted a general interest on circulating miRs as potential bedside biomarkers for detecting a wide range of cardiovascular diseases and for monitoring disease progression and efficacy of therapeutic regimens (Wang et al. 2013a; Villar et al. 2011, 2013; Garcia et al. 2013; for a review see Creemers et al. 2012).

Circulating miRs are released from cells incorporated into extracellular vesicles (exosomes and microvesicles) or bound to RNA-binding proteins such as argonaute or lipoprotein complexes such as high-density lipoproteins (HDL), etc (Valadi et al. 2007). These types of complexings provide protection against degradation by the endogenous RNAse activity (reviewed in: Creemers et al. 2012). Identification of exosomes containing miRs, which are functional upon delivery to the recipient cells, has unveiled a new role for miRs as juxtacrine/paracrine/endocrine mediators of cell-to-cell communication, involved in the transfer of genetic information between neighboring cells or cells located at distant organs (Kuwabara et al. 2011). As an example, cell-specific inflammatory exosomes (released from platelets, endothelial cells, and monocyte or monocyte-derived cells) are enriched in a subset of miRs that are functionally involved in the pathogenesis of cardiovascular diseases and whose circulating levels have been proposed as putative biomarkers of such diseases (Hulsmans and Holvoet 2013). Exosomes also constitute a novel therapeutic strategy for delivering cargos of miRs, RNAs, or even drugs (Fleury et al. 2014).

Modulation of MicroRNA Activity

miR dysregulation is a common feature in cancer, central nervous system disorders, inflammation, cardiovascular diseases, and metabolic disorders. The manipulation of miR expression is an attractive tool for therapeutic intervention, and several miR candidates have already progressed into clinical development (van Rooij and Olson 2012). There are two approaches to developing miR-based therapeutics: miR antagonists and miR mimics (reviewed in: Dangwal and Thum 2014; van Rooij and Kauppinen 2014). miR mimics are used to restore the level of a downregulated miR during disease progression. Delivery of miR replacement therapies in vivo was accomplished using lentiviral or adenoviral constructs expressing the miR of interest, miR-liposomal formulations, miR-polyethyleneimine or miR-atelocollagen complexes, etc.

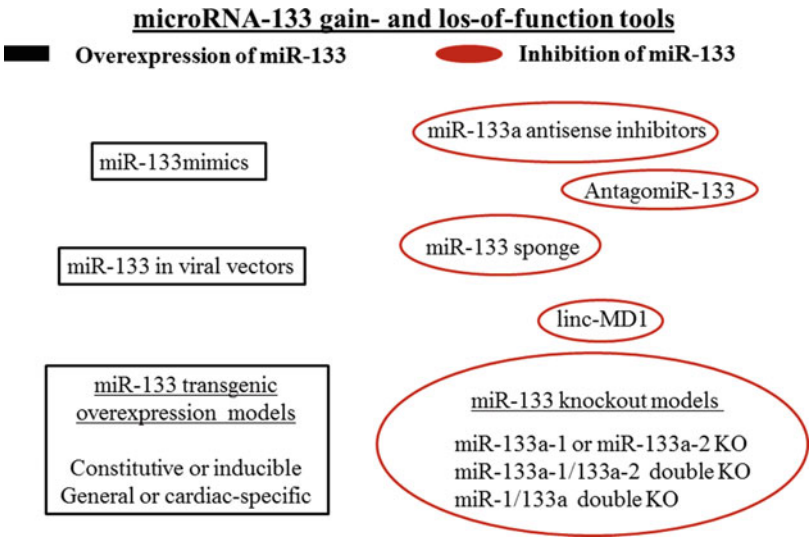

Fig. 2 MicroRNA-133 gain- and loss-of-function strategies

The objective of using miR antagonists is to inhibit endogenous miRs that are upregulated in a particular disease process. The function of mature miRs can be blocked using either anti-miRs or miR sponges. Anti-miRs are synthetic single-stranded antisense oligonucleotides that hybridize by Watson-Crick base pairing to target miRs and block their function. miR sponges are synthetic RNA molecules harboring complementary binding sites to the seed sequences of a given miR or a miR family. The miRs of interest that bind to the sponge become unavailable to bind their targets. Chemical modification strategies of the oligonucleotides, including 2′-O-methylation or locked nucleic acid (LNA) modification, have been developed to improve miR binding affinity and biostability. Cholesterol conjugation of anti-miRs (antagomiRs) improves their cellular uptake.

Modulation of miR activity has proven useful as experimental tool (Dangwal and Thum 2014; van Rooij and Kauppinen 2014). Gain- and loss-of-function approaches (Fig. 2) have been applied to ascertain processes regulated by miR-133 in the cardiovascular system, during the embryonary period and in adulthood. Classical knockout technology allowed unraveling the role of miR-133 and miR clusters in cardiac development using mice deficient for either miR-133a-1 or miR-133a-2 or both (Liu et al. 2008) and mice with targeted inactivation of miR-1/133a clusters (Wystub et al. 2013). The combination of experimental models of human disorders with miR-133 activity modulation provided insights on the role played by this miR and their targets in cardiovascular pathologies. For example, the pathogenetic role miR-133 in murine hypertrophic cardiomyopathy was demonstrated in gain- and loss-of-function studies using adenoviral vectors containing a miR-133 mimic and specific miR-133 sponges, respectively (Caré et al. 2007). Also, a modulatory role for miR-133 on myocardial fibrosis and apoptosis was demonstrated using mouse models of cardiac-specific miR-133a transgenic overexpression (Castaldi

et al. 2014). Similar approaches were used to demonstrate the participation of miR-133 in murine models of arterial wall injury and atherosclerosis (Torella et al. 2011; Gao et al. 2014).

The muscle-specific lnc-RNA, linc-MD1, constitutes an example of ceRNA which governs muscle differentiation by targeting both miR-133 and miR-135. linc-MD1 prevents miR-133 and miR-135 to regulate the expression of transcription factors that activate muscle-specific gene expression. Downregulation or overexpression of linc-MD1 correlates with retardation or anticipation of the muscle differentiation program, respectively (Cesana et al. 2011).

MicroRNA-133 Taxonomy and Targets

miR-133 was first characterized in mice and, since then, homologues have been discovered in the genomes of nonmammalian vertebrates and in many mammalian species, including the *Homo sapiens* (see miRbase). miR-133's presence in the miRNome has been firmly established in health and disease, suggesting that it plays similar functions in different organisms. There are three known miR-133 human genes: two gene loci (bicistronic) for miR-133a and one for miR-133b (miR map). The first location of a miR-133a precursor is intronic (Mib1 gene) in chromosome 18 (18q11.2), in a sequence of 88 nucleotides (GGGAGCCAAAUGCUUUGCUAGAGCUGGUAAAAUGGAACCAAAUCGACUG UCCAAUGGAUUUGGUCCCCUUCAACCAGCUGUAGCUGUGCAUUGAUGGCG CCG) called miR-133a-1 stem loop or pre-miR-133a-1. The catalytic cleavage by DICER of pre-miR-133a-1 gives rise to miR-133a-1-3p, which contains the 53–74 bp (UUUGGUCCCCUUCAACCAGCUG) from the 3′ arm and to the marginally co-expressed miR-133-1-5p, formerly called miR-133a*, which corresponds to the 5′arm 16–37 bp (AGCUGGUAAAAUGGAACCAAAU). Although miR-133-1-5p degradation after its separation from the duplex was reported, today both forms of miR-133 are considered mature functional molecules, and bioinformatic and experimental studies are unraveling their targets. A different additional precursor of miR-133a, called pre-miR-133a-2, is also intronic (putative uncharacterized protein C20orf166 gene) in chromosome 20. Its sequence (GGGAGCCAAAUGCUUUGCUAGAGCUGGUAAAAUGGAACCAAAUCGACUG UCCAAUGGAUUUGGUCCCCUUCAACCAGCUGUAGCUGUGCAUUGAUGGCG CCG) also contains the previously described 3p and 5p miR-133a sequences that, though identical, were called miR-133a-2-3p and miR-133a-2-5p (Fig. 3). The third miR-133 is miR-133b (UUUGGUCCCCUUCAACCAGCUA). Its mature form comes from the 3′arm of pre-miR-133b (CCUCAGAAGAAA GAUGCCCCCUGCUCUGGCUGGUCAAACGGAACCAAGU CCGUCUUCCUGAGAGGUUUGGUCCCCUUCAACCAGCUACAGCAGGGCUGG CAAUGCC CAGUCCUUGGAGA) which is located in chromosome 6.

The members of the miR-133 family differ depending on the species. The human family of miR-133 includes miR-133a-3p, miR-133a-5p, and miR-133b. In mice and

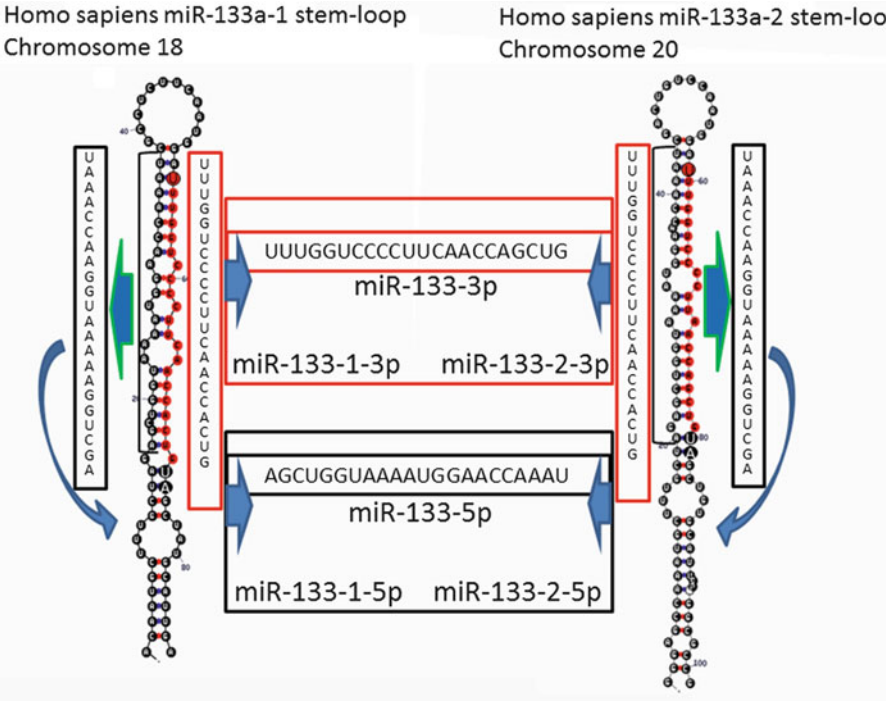

Fig. 3 The *Homo sapiens* microRNA-133a family. There are two gene loci (bicistronic) for miR-133a located in chromosome 18 (miR-133a-1) and 20 (miR-133a-2). The sequence of the precursor pre-miR-133a-1 (stem loop of the *left*) includes two mature molecules of miR-133a: the major species miR-133a-1-3p (depicted in *red*) is derived from the 3′ arm; the minority miR-133-1-5p is derived from the 5′arm (in *black*). The bicistronic precursor pre-miR-133a-2 (stem loop of the *right*) contains two miR-133a sequences that are identical to the former but, in this case, are called miR-133a-2-3p and miR-133a-2-5p (Modified from miRNA map: http://mirnamap.mbc.nctu.edu.tw)

rats, there are five different miR-133 members: miR-133a-3p, miR-133a-5p, 133b-3p, miR-133b-5p, and miR-133c. In all species, miR-133a-3p is the most abundantly expressed (see miRSearch V3.0).

The presence of multiple miRs with identical or similar mature sequences is a common and evolutionary conserved feature, indicating functional significance. Both miR-133 and miR-1 are muscle-specific miRs (myomiRs) that are found in bicistronic position in the mammalian genome and are transcribed together. miR-1 and miR-133a are even encoded by duplicated bicistronic loci (miR-1-1/miR-133a-2 and miR-1-2/miR-133a-1) with identical sequences of the mature miRs. There is another bicistronic cluster encoding the nearly identical miR-206/miR-133b (Wystub et al. 2013).

Up-to-date, the combination of computational analysis and experimental approaches revealed that miR-133a-3p has 399 predicted targets, 42 of which have

been validated experimentally. A non-comprehensive list of validated targets includes the following: collagen type I α1, fibrilin, tropomyosin, TGF-β1, TGF-βRII, MEF-2, HCN2, KCNQ1, ERG, NFATc4, RhoA, Runx2, and PITX3.

Embryogenesis, Myocardial Regeneration, and Cell Reprogramming

miR-133a is muscle specific, expressed by skeletal and cardiac striated myocytes, and participates in various physiological and pathological phenomena (Mooren et al. 2014; Thum et al. 2007; Condorelli et al. 2014). Together with miR-1 represses non-muscle genes in human and murine embryonic stem cells, favoring mesoderm formation and modulating their differentiation into muscle tissue (Ivey et al. 2008). In the heart, miR-133 is exclusively expressed by cardiomyocytes (Townley-Tilson et al. 2010) and the miR-133a-1/miR-1-2 and miR-133a-2/miR-1-1 clusters play a significant role in the maturation of embryonic cardiomyocytes to more differentiated fetal cardiomyocytes through the adjustment of the levels of myocardin (Wystub et al. 2013). These miRs are direct transcriptional targets of several muscle differentiation regulators, the most important of which is serum response factor (SRF), which points to the existence of a common set of control elements that modulate the development of cardiac and skeletal muscle (Zhao et al. 2005). miR-133a plays also an indispensable role in cardiomyocyte proliferation and ventricular septation during embryonic cardiac development. The lack of miR-133a results in ectopic activation of smooth muscle genes in the heart and aberrant cardiomyocyte proliferation, whereas its overexpression derives in a low cardiomyocyte proliferation with hypotrophic organogenesis and loss of embryonic viability (Liu et al. 2008). Overexpression of miR-133 by transgenesis in zebra fish hampers myocardial regeneration after cardiac apex avulsion, and, conversely, miR-133 depletion enhances cardiomyocyte proliferation and speeds up the regenerative healing after apical severance in this model (Yin et al. 2012). These findings led Yin et al. to suggest that cardiac regeneration in the adult zebra fish is evolutionarily related to myocardial hypertrophy in mammals and that miR-133 downregulation is a remnant shared by both remodeling phenomena.

Recent studies demonstrate that miR-133a, together with the combination of Gata-binding protein 4, Hand2, T-box5 and myocardin (Nam et al. 2013), or Gata4 plus Mef2c and Tbx5 (GMT), or GMT plus Mesp1 and myocardin (Muraoka et al. 2014), is able to promote the reprogramming of neonatal and adult mouse or human fibroblasts to cardiac-like myocytes. In addition, transplantation of cardiac progenitor cells overexpressing miR-133a improves the heart function in a rat model of myocardial infarction by increasing cardiomyocyte proliferation and vascularization and reducing cardiomyocyte apoptosis and myocardial fibrosis (Izarra et al. 2014).

Pathologic Cardiac Remodeling

There is abundant evidence of miR-133a involvement in pathological forms of cardiac plasticity. The reappearance in adulthood of the embryonic gene expression pattern, characterized by diminished expression of the miR-133 cluster, would be teleonomically sound, as programs for cardiac growth experiment a robust activation in response to the demands imposed by either the somatic development or a pathologic cardiac mechanical overload (Table 1) (Thum et al. 2007).

Cardiac miR microarray analyses in rodent models of cardiac hypertrophy show a reduction in miR-133a expression coincident with an increase in cardiac mass (van Rooij et al. 2006; Ye et al. 2013). Validation of these data with northern blotting or qRT-PCR confirms a decrease in the cardiac levels of miR-133 in exercised rats, in mice subjected to transverse aortic constriction (TAC), in several transgenic mice models of LV hypertrophy, and in human LV from patients with pathologies that promote hypertrophic growth (van Rooij et al. 2006; Carè et al. 2007). In the mouse model of TAC, miR-133 cardiac expression is reduced 1 week after surgery but recovers basal levels at 3 weeks, time at which LV mass typically plateaus with this model (Matkovich et al. 2010). These observations have been confirmed with functional in vitro and in vivo experiments of gain and loss of function. Overexpression of miR-133 with adenoviral vectors in cultured neonatal murine cardiomyocytes results in significant blunting of the hypertrophic hallmark features induced by phenylephrine or endothelin-1 treatments. Conversely, molecular sequestration of miR-133a, using adenoviral vectors carrying MREs that act as a decoy and silence the miR, results in evolution toward a hypertrophic phenotype of fetal or adult mouse cardiomyocytes in culture (Carè et al. 2007). Also, mice treated subcutaneously or transcoronary with an antagomiR targeted to miR-133a showed reactivation of the fetal gene expression profile, activation of the cardiac hypertrophic program, and significant increases in LV wall thicknesses and total cardiac mass. On the other hand, overexpression of miR-133 in Akt transgenic mice, a model of cardiac hypertrophy, resulted in volume reduction of cardiomyocytes and reduced expression of fetal genes (Carè et al. 2007).

Not all the data on the role of miR-133 in hypertrophy models are univocal though. Overexpression of this miR in transgenic mice does not palliate the increase in cardiac mass observed after TAC, which is similar to their wild-type littermates even though the transgenic animals, contrary to the wild-type, do not exhibit a postoperative reduction in miR-133 expression levels. Also, miR-133 expression is not reduced in some models of genetically induced cardiac hypertrophy (Matkovich et al. 2010).

The plasma levels of the muscle-related miRs are altered in humans in response to both acute aerobic and endurance exercise. In athletic runners, exercise induced an increase in the circulating levels of miR-133a which exhibit positive correlations with the maximal oxygen uptake, with the running speed at individual anaerobic lactate threshold, and with the cardiac interventricular septal thickness (Mooren et al. 2014). At the other end, unloading the heart in patients with advanced heart

Table 1 miR-133 in patients with cardiac disease

Pathology	Cohorts	miR-133 regulation	Other major findings	References
Aortic stenosis	Patients with moderate to severe AS ($n = 112$) and healthy controls ($n = 40$)	↓miR-133a qRT-PCR Plasma samples	miR-1 and miR-378 reduced in AS miR-378 is a predictor of LVH	Chen et al. (2014)
	Patients with isolated AS ($n = 9$) and nonhypertrophic controls ($n = 4$)	↓miR-133a in AS qRT-PCR Myocardial samples	↓miR-30c CTGF: target of miR-133a; overexpressed in AS	Duisters et al. (2009)
	AS patients ($n = 46$), surgical controls with no pressure or volume overload ($n = 23$)	↓miR-133a in patients with persistent LVH 1 year after aortic valve replacement qRT-PCR Myocardial samples	Myocardial miR-133a: predictor of postoperative LV mass loss and mass normalization one year after surgery	Villar et al. (2011)
	AS patients ($n = 74$)	↓circulating miR-133a in patients with persistent LVH 1 year after aortic valve replacement qRT-PCR Myocardial samples Plasma samples: Peripheral and coronary sinus blood	Circulating miR-133a is a predictor of postoperative LV mass loss and of mass normalization one year after surgery. The heart is a main contributor to circulating miR-133a	García et al. (2013)
Hypertrophic cardiomyopathy	HCM patients ($n = 4$) and controls ($n = 2$) Patients with mitral stenosis and atrial dilatation ($n = 3$), and controls ($n = 3$)	↓miR-133 Northern blot Myocardial samples		Carè et al. (2007)
	HCM patients ($n = 41$) and age and sex matched healthy subjects ($n = 41$)	↑miR-133a in plasma qRT-PCR Plasma samples	miRs -199a-5p, -27a, and -29a correlated with hypertrophy and miR-29a correlated also with fibrosis	Roncarati et al. (2014)

(*continued*)

Table 1 (continued)

Pathology	Cohorts	miR-133 regulation	Other major findings	References
Left ventricular hypertrophy	ICM ($n = 19$), DCM ($n = 25$), and AS ($n = 13$) patients and controls ($n = 10$)	miR-133a: no change miR high-throughput bead-based platform qRT-PCR Myocardial samples	↑miR-214 in all pathological groups ↓miR-19a and -19b in DCM and AS Distinct miR profiles in patients with different diagnosis	Ikeda et al. (2007)
Heart failure	LVs with DCM ($n = 6$), normal adult LVs ($n = 4$) and fetal hearts ($n = 6$)	miR-133: no change miR and mRNA microarrays qRT-PCR Myocardial samples	Striking similarity of miR and mRNA expression profiles in HF and fetal hearts as compared with adult healthy hearts	Thum et al. (2007)
LV mechanical circulatory support	Patients with cardiomyopathy ($n = 17$) supported with LVAD	During LVAD: ↓miR-133a in DCM and ↑miR-133a in ICM qRT-PCR Myocardial samples	Similar trends for miR-1 and miR-133b	Schipper et al. (2008)
	Patients with congestive HF with ($n = 10$: 4 ischemic; 6 nonischemic) or without ($n = 17$: 7 ischemic; 10 nonischemic) LVAD. Nonfailing hearts ($n = 11$)	↑miR-133 in HF versus non-HF and normalizes with LVAD miR-PCR-array Myocardial samples	Changes in miR signature more sensitive than mRNA profile in HF. Changes in miRs nearly normalized with LVAD	Matkovich et al. (2009)
	Patients with DCM ($n = 28$) supported with LVAD: 14 support dependent and 14 experienced LV recovery. Test cohort: $n = 14$; Validation cohort: $n = 14$. Control nonfailing hearts ($n = 7$)	miR-133: no change miR-PCR-array qRT-PCR Myocardial samples	Pre-support expression of miR-23a and -195 differs between hearts that recovered and hearts that did not	Ramani et al. (2011)

(*continued*)

Table 1 (continued)

Pathology	Cohorts	miR-133 regulation	Other major findings	References
	Patients with ICM ($n = 10$) and DCM ($n = 9$) supported with LVAD	miR-133: no change miR-PCR-array qRT-PCR Plasma samples Myocardial samples	↑circulating miR-483-3p with LVAD Baseline miR-1202 identified good and bad LVAD responders	Morley-Smith et al. (2014)
	Paired samples of failing LVs with ICM ($n = 8$) and DCM ($n = 8$) before and after support with LVAD. Nonfailing LVs ($n = 8$)	No specific reference to miR-133 Next-generation sequencing of RNA Myocardial samples	lncRNA profiles discriminate better different HF pathologies than mRNA or miR signatures. lncRNAs profile improves more with LVAD support than either mRNAs or miRs	Yang et al. (2014)
	Failing LVs with ICM ($n = 13$) and DCM ($n = 21$) before and after support with LVAD. Nonfailing adult LVs ($n = 8$) and fetal hearts ($n = 5$)	miR-133: no change Small RNA sequencing Myocardial samples Plasma and serum samples	↑circulating myomiRs in advanced HF and nearly normalized 3 months after LVAD support	Akat et al. (2014)

AS valvular aortic stenosis, *CTGF* connective tissue growth factor, *DCM* dilated cardiomyopathy, *HF* heart failure, *HCM* hypertrophic cardiomyopathy, *ICM* ischemic cardiomyopathy, *lncRNA* long noncoding RNA, *LVAD* left ventricular assist device, *LVH* left ventricular hypertrophy

failure under mechanical circulatory support also modifies the expression of miR-133a but in different direction, depending on the etiology of the cardiac underlying pathology. Thus, in patients supported with a LV assist device, the myocardial expression of miR-133a decreases if their underlying pathology is an idiopathic dilated cardiomyopathy or increases if it is of ischemic origin (Schipper et al. 2008). This observation, however, is at variance with the findings of other groups (Ramani et al. 2011; Morley-Smith et al. 2014) who did not find differences in miR-133a cardiac or plasma levels in heart failure patients after support with a mechanical LV assist device (Table 1).

The prognostic potential of myocardial and/or circulating miR signatures for the estimation of cardiac recoverability in patients with advanced heart failure under mechanical circulatory support is currently under active investigation. miRNomic profiling appears in this respect more promising than transcriptomic analysis. mRNA levels feature little expression changes in these patients, as compared with controls

with stable heart failure, whereas myocardial miR levels undergo generalized and profound changes both in patients with advanced heart failure and in patients treated successfully with mechanical means (Table 1) (Matkovich et al. 2009).

Reports on human cardiac pathologies which associate LV hypertrophy described downregulation of myocardial and/or circulating miR-133a often in association with miR-1 (Caré et al. 2007; Chen et al. 2014), and this phenomenon has been partially pinpointed in microarray analyses (Table 1) (Ikeda et al. 2007). In patients with pure, severe, a valvular aortic stenosis, the myocardial expression and circulating levels of miR-133 were found significantly reduced as compared with surgical controls without pressure or volume overload (Villar et al. 2011; García et al. 2013). In these patients, undergoing aortic valve replacement, both myocardial and circulating miR-133a preoperative levels were predictors of the quantitative LV mass loss and the probability of mass normalization 1 year after surgery. Interestingly, the postoperative increase in aortic valve area, as a surrogate of the reduction in pressure overload achieved by these patients, did not arise as a predictor of these variables, suggesting that in the clinical arena the mechanical stress imposed by the valve pathology acts as a trigger of the cardiac remodeling process, but there are other important players that have major roles in the regression of hypertrophy (Table 1) (Villar et al. 2011; García et al. 2013).

As to the origin of miR-133a in the circulation, there are several potential candidates: striated skeletal muscle, vascular wall, blood elements, etc. It has been shown, however, that in patients with severe aortic stenosis, there is a positive concentration gradient between the coronary outflow and the systemic circulation and a significant positive correlation between the miR levels at both spots and with the myocardial expression levels at the LV (García et al. 2013). These observations support the role of the heart as a key contributor to the miR-133a circulating levels in this context.

It is not clear whether miR-133 participates in the maladaptive cardiac remodeling that accompanies human end-stage heart failure. Indeed, transcriptional profiling of human myocardium with microarrays or next-generation sequencing have shown its lack of regulation in this scenario (Table 1) (Thum et al. 2007; Ikeda et al. 2007; Matkovich et al. 2009; Yang et al. 2014; Akat et al. 2014).

Fibrotic Remodeling of the Heart and Apoptosis

There is an ample consensus on the anti-fibrotic role of miR-133a in the cardiac stress condition. The controversy persists, though, on the precise mechanistic elements whereby fibrotic remodeling is blunted by this miR in different pathologic clinical or experimental situations. In rodent models of pressure overload and in patients with severe valvular aortic stenosis, Duisters et al. (2009) found that myocardial levels of miR-133 correlated inversely with the expression of connective tissue growth factor (CTGF), a potent inducer of extracellular matrix production acting downstream of transforming growth factor-β (TGF-β) (Table 1). Gain- and loss-of-function experiments showed that the expression levels of miR-133 correlated inversely with the levels of CTGF in cultured cardiomyocytes and with CTGF and extracellular matrix elements in fibroblasts. Luciferase reporter assays confirmed

that CTGF and the profibrogenic cytokine TGF-β1 and the TGF-β type II receptor were direct targets of miR-133 (Duisters et al. 2009; Shan et al. 2009). A discordant finding in this respect is the observation of increased mRNA CTGF levels after aortic banding in transgenic mice overexpressing miR-133a (Matkovich et al. 2010).

In a rat model of angiotensin-dependent systemic hypertension, it has been shown that gene and protein expression levels of collagen I A1 evolve with a significant inverse correlation with those of miR-133a. The bioinformatic prediction algorithm studies of putative binding sites and luciferase reporter assays confirmed collagen I A1 as a direct target of miR-133a (Castoldi et al. 2012). Other direct and indirect mechanisms of the antifibrotic role of miR-133a in cardiac stress act via inhibition of caspase-mediated cardiomyocyte apoptosis (Matkovich et al. 2010).

There is experimental in vivo evidence that, after an ischemic insult, miR-133a has a beneficial effect on the myocardial cell fate by targeting caspase-9 and the consequent reduction in apoptosis. Analyzing the participation of miRs in the salutary effects of myocardial ischemic postconditioning in a rat model of ischemia/reperfusion, it has been shown that postconditioning, as compared with simple ischemia/reperfusion, was accompanied by overexpression of miR-133a, reduction in the number of TUNEL-positive cells and in caspase-9 expression. Cardiac intramuscular pretreatment of the animals with a miR-133a mimic or an anti-miR oligonucleotide prevented or enhanced, respectively, the proapoptotic effect of ischemia/reperfusion, while blunting or increasing the expression of caspase-9. This cytoprotective effect of miR-133a was also duplicated in primary rat neonatal cardiomyocytes subjected to ischemia/reperfusion (He et al. 2011).

Recent data obtained with a novel cardiac-specific TetON-miR-133 inducible transgenic mouse model show that overexpression of miR-133 in vivo results in a significant reduction of myocardial fibrosis and cell apoptosis after TAC in these animals as compared with wild-type littermates (Castaldi et al. 2014). The authors show proof of direct targeting in the myocardium of the β_1-adrenergic signaling pathway at multiple levels (β_1-adrenergic receptor, adenylate cyclase VI, catalytic subunit β of cAMP-dependent protein kinase A, and exchange protein activated by cAMP or EPAC) by miR-133 that would explain the anti-apoptotic effect observed in their transgenic animals after aortic constriction (Castaldi et al. 2014).

Ablation of the expressions of both miR-133a-1 and miR-133a-2 in double knockout mice resulted in a high lethality, but the approximately 24 % of animals that survived to adulthood displayed extensive myocardial fibrosis and systolic dysfunction and evolved toward dilated cardiomyopathy at age 5–6 months with a high incidence of sudden death (Liu et al. 2008; Da Costa Martins and De Windt 2012).

Coronary Artery Disease

Acute coronary syndrome (ACS), which includes ST-elevation myocardial infarction (STEMI), non-ST-elevation myocardial infarction (NSTEMI), and unstable angina (UA), is the leading cause of death worldwide. Currently, cardiac troponins

are the most commonly used biomarkers for myocardial damage in this context, but their clinical value is limited in many cases.

Several publications evidenced that a number of miRs are differentially regulated in patients with coronary heart disease (CHD) and investigated the usefulness of their circulating levels as prognostic and diagnostic biomarkers (reviewed in Fichtlscherer et al. 2011; Fiedler and Thum 2013; Condorelli et al. 2014). Herein, we summarize up-to-date research dealing with the potential value of miR-133 in this entity (Table 2).

Experimental studies using models of acute myocardial infarction (AMI) in mice showed significant downregulation of miR-133a and miR-133b in the damaged myocardium in comparison with control animals (D'Alessandra et al. 2010; Kuwabara et al. 2011). The clinical relevance of these findings was supported by studies that, using autopsy samples from AMI patients, confirmed that the expression of both miRs was subjected to downregulation in the infarcted and border zones in comparison with non-infarcted myocardium or from transplant donors (Kuwabara et al. 2011; Boštjančič et al. 2010, 2012).

Opposite in direction, circulating levels of miR-133a/b exhibited early elevations after coronary occlusion in experimental models of AMI in mice (D'Alessandra et al. 2010), rats (Wang et al. 2010), and pigs (Gidlof et al. 2011), with a time course that closely paralleled the increase in cardiac troponin I (cTnI). The specificity of these features was supported by the lack of changes in circulating miRs following skeletal muscle damage induced by acute hind-limb ischemia (D'Alessandra et al. 2010).

The time course of circulating miRs after acute myocardial ischemia has been studied after clinical transcoronary interventricular septal ablation in patients with hypertrophic obstructive cardiomyopathy (Liebetrau et al. 2013). This therapeutic maneuver reproduces an experimental myocardial infarction that makes it particularly suitable to assess with precision the initial phases of cardiac miR release to the circulation after induction of acute ischemia. An added advantage is that these patients do not have previous coronary artery disease that may alter acute miR release by a preexistent ischemic preconditioning. Plasma concentrations of both miR-1 and miR-133a rose consistently (100 % of patients) and very early (15 min) after induction of ischemia. miR-133a peaked 75 min after coronary occlusion, when its circulating level was over 50-fold the control value, and declined between the fourth and the eighth hour but still was increased (30-fold change) 24 h after induction of ischemia (Liebetrau et al. 2013). These results demonstrate a very quick and very sensitive increase in miR-133a after acute myocardial ischemia that is comparable, and perhaps confirmatory, of cTnT shifts.

In the clinical setting, elevated serum/plasma levels of miR-133a and/or miR-133b were consistently reported by a number of studies assessing the value of miR-133 as diagnosis biomarker in patients with ACS (Corsten et al. 2010; D'Alessandra et al. 2010; Wang et al. 2010; DeRosa et al. 2011; Fichtlscherer et al. 2010; Gildlöf et al. 2011; Kuwabara et al. 2011; Zile et al. 2011; Devaux et al. 2013; Wang et al. 2013b; Yao et al. 2014), as well as in patients with stable CHD (Fichtlscherer et al. 2010). Of note, the circulating levels of miR-133a/b in MI patients exhibited positive correlations with the extent of myocardial damage as

Table 2 microRNA-133 in patients with coronary arterial disease

Pathology	Cohorts	miR-133 regulation	Other major findings	References
Stable coronary disease (SCAD)	Derivation cohort: SCAD patients ($n = 36$) and healthy volunteers ($n = 17$) Validation cohort: SCAD patients ($n = 31$) and healthy volunteers ($n = 14$)	↑miR-133a (derivation cohort); no change (validation cohort) qRT-PCR Plasma	↓circulating miR-126, -17, -92a, -145, -155 ↑miR-208a	Fichtlscherer et al. (2010)
Acute coronary syndrome (ACS)	AMI patients ($n = 93$) and healthy subjects ($n = 66$)	No change miR-133a/b qRT-PCR Plasma	↑miR-1 in AMI; normalization at discharge	Ai et al. (2010)
	AMI ($n = 50$) patients and healthy trauma victims ($n = 8$)	↓miR-133a qRT-PCR Myocardium	↓miR-1 and ↑miR-208 in infarct tissue	Boštjančič et al. (2010)
	AMI patients ($n = 32$) and control subjects ($n = 36$)	↑miR-133 qRT-PCR Plasma	miR-208b and -499 correlated with cTnT and CPK	Corsten et al. (2010)
	STEMI patients ($n = 33$) and healthy subjects ($n = 7$)	↑miR-133a/b Microarray, qRT-PCR Serum	↑miR-1, -499-5p ↓miR-122 and -375 Time courses of miR-1 and miR-133a/b similar to cTnI	D'Alessandra et al. (2010)
	AMI patients ($n = 33$) and healthy subjects ($n = 30$)	↑miR-133 qRT-PCR Plasma	↑miR-208a miR-133a correlated with cTnI ROC curve: plasma miR-133a and miR-208a sensitive predictors for CAD miR-208a highest sensitivity and specificity	Wang et al. (2010)
	No CAD ($n = 7$), SCAD ($n = 31$), and ACS ($n = 19$) patients	↑miR-133a qRT-PCR Aortic and coronary sinus plasma	↑miR-133a,-208a,-126,-92a, and -155 ↑miR-133a and -499 transcoronary gradients ↓miR-126 transcoronary gradient	DeRosa et al. (2011)
	STEMI patients ($n = 26$) and healthy subjects ($n = 11$)	↑miR-133a qRT-PCR Plasma	↑ miR-1, -133a, -208b, and -499-5p within 12 h of the onset of MI symptoms miR-1 and miR-133 detectable in urine	Gildlof et al. (2011)

(*continued*)

Table 2 (continued)

Pathology	Cohorts	miR-133 regulation	Other major findings	References
	ACS patients ($n = 444$)	miR-133a STEMI > NSTEMI qRT-PCR Plasma	↑miR-133a,-208: ↑mortality risk	Widera et al. (2011)
	AMI patients ($n = 12$) and age-matched subjects ($n = 12$)	↑miR-133 qRT-PCR Plasma	Time courses of plasma miR-21, -29a, -133a, and -208 during 90 days after AMI	Zile et al. (2011)
	AMI ($n = 6$) patients who died one week after MI	↓miR-133a/b Microarray, qRT-PCR Infarct versus remote myocardium	↓ SERCA2 in infarcted tissue 43 differentially expressed miRs Bioinformatic prediction of SERCA targeting by regulated miRs	Boštjančič et al. (2012)
	STEMI patients ($n = 216$) undergoing primary angioplasty	↑miR-133a qRT-PCR Plasma	Prognostic information of major cardiovascular events within 6 months after AMI	Eitel et al. (2012)
	ACS ($n = 29$) and non-ACS ($n = 42$)	↑miR-133a qRT-PCR Plasma	↑miR-1 miR-1 and miR-133a correlated with cTn	Kuwabara et al. (2011)
	AMI patients ($n = 246$) and control subjects ($n = 127$)	Unchanged miR-133a qRT-PCR Plasma	miR-133a and -423-5p stable from baseline to 1-year follow-up	Bauters et al. (2013)
	1115 patients with chest pain (AMI = 224)	↑miR-133a in AMI patients qRT-PCR Plasma	↑miR-208b, -499, and -320a miR-208b: highest diagnostic accuracy for AMI	Devaux et al. (2013)
	AMI patients ($n = 13$), angina pectoris patients ($n = 176$), and control subjects ($n = 127$)	↑miR-133a qRT-PCR Plasma	miR-133a correlated with cTnI in AMI patients miR-133a: sensitive predictor for CAD	Wang et al. (2013b)
	TTC ($n = 36$), STEMI ($n = 27$) patients, and healthy subjects ($n = 28$)	↑miR-133a in TTC and STEMI qRT-PCR Plasma	Unique signature including miR-1, -16, -26a, and -133a differentiated TTC from AMI	Jaguszewski et al. (2014)

(*continued*)

Table 2 (continued)

Pathology	Cohorts	miR-133 regulation	Other major findings	References
	STEMI ($n = 25$), NSTEMI ($n = 51$), and non-AMI patients ($n = 110$)	↑miR-133 STEMI and NSTEMI qRT-PCR Plasma	miR-133 in STEMI > NSTEMI	Peng et al. (2014)
	Patients undergoing coronary angiography with abnormal ($n = 26$) and normal ($n = 22$) endothelial function	↑miR-133a in coronary sinus versus aorta qRT-PCR Plasma	↑transcoronary gradients of miR-92a and miR-133 in patients with CED	Widmer et al. (2014)
	CABG patients with ($n = 28$) and without ($n = 89$) perioperative MI	↑miR-133 qRT-PCR Plasma	miR-133a levels peaked earlier and correlated with cTnI	Yao et al. (2014)

CABG coronary artery bypass graft, *CED* coronary endothelial dysfunction, *cTnI* cardiac Troponin I, *cTnT* cardiac Troponin T, *MI* myocardial infarction, *ROC* receiver operating characteristic, *STEMI* ST-segment elevation MI, *TTC* Takotsubo cardiomyopathy

assessed by cTnI or cTnT and achieved an earlier concentration peak (D'Alessandra et al. 2010; Wang et al. 2010; Widera et al. 2011; Kuwabara et al. 2011; Wang et al. 2013b; Yao et al. 2014). Also, receiver operating characteristic (ROC) analysis further indicated that miR-133 might be a sensitive biomarker for ACS diagnosis (Wang et al. 2010; Kuwabara et al. 2011; Yao et al. 2014). In a recent meta-analysis of 19 articles, Cheng et al. (2014) reviewed literature dealing with the association between circulating miRs and myocardial infarction. Four of these studies, including 285 patients, dealt with miR-133a. The summary ROC curves of the meta-analysis indicated that miR-133a [sensitivity, 0.89 (95 % CI, 0.83–0.94; $P = 0.0047$); specificity, 0.87 (95 % CI, 0.79–0.92; $P = 0.026$)] may be suitable as diagnostic biomarker of MI (Cheng et al. 2014).

Increased circulating levels of miR-133a across the coronary circulation have been reported in patients with early coronary atherosclerosis (manifested by coronary microvascular endothelial dysfunction), patients with stable CHD, and cTn-positive ACS patients (Fichtlscherer et al. 2010; De Rosa et al. 2011; Widmer et al. 2014). These findings suggest a release of miR-133 from cardiomyocytes into the coronary circulation during myocardial injury as part of the first steps of the CHD and support circulating level of miR-133 as a sensitive surrogate marker for progression of atherosclerosis in the coronary tree, even in its initial phases.

A specific signature of miRs dysregulated in plasma, including miR-133a, miR-1, miR-16, and miR-26a, has been identified in Takotsubo disease, a cardiomyopathy with an acute phase clinically indistinguishable from AMI (Jaguszewski et al. 2014).

However, a previous study (Kuwabara et al. 2011) reported no specific profile in the circulating levels of miR-133a in either UA or Takotsubo cardiomyopathy.

In a series of 246 patients, Bauters et al. (2013) reported that miR-133a circulating levels remained stable from baseline to 1 year after AMI and lacked any relationship with cardiac biomarkers, including B-type natriuretic peptide, C-reactive protein, and cTnI, or with LV remodeling during the year following the acute episode.

In STEMI patients treated with percutaneous coronary intervention, higher circulating levels of miR-133a associated larger infarcts, more severe reperfusion injury, and decreased myocardial salvage, as determined by cardiac magnetic resonance. Circulating miR-133a constituted an independent positive risk factor for death or other major adverse cardiovascular events within 6 months after AMI (Widera et al. 2011; Eitel et al. 2012). However, miR-133a concentrations did not add prognostic information to troponins. In patients with angiographically documented CHD, Wang et al. (2013b) reported a significant positive correlation of circulating miR-133a levels with the severity of coronary lesions and constituted a sensitive independent predictor for coronary disease.

Peripheral Vascular Disease

There is growing evidence for a pivotal role of the plasticity and phenotypic switching of vascular smooth muscle cells (VSMCs) in vascular diseases. Adult VSMCs are normally quiescent and programmed for contraction, but they maintain remarkable plasticity and, depending on the signals present in their local environment, can acquire dedifferentiated phenotypes with increased ability to migrate, proliferate, and promote extracellular matrix production, inflammatory signals, and calcification. VSMC phenotypic switch plays a critical role in vascular repair. Dysregulation of this plasticity program contributes to the alterations of the arterial wall architecture, called remodeling, which can be found in several vascular disorders in humans, such as essential hypertension, atherosclerosis, vascular calcification, aneurysmal disease, or restenosis (Lacolley et al. 2012; Condorelli et al. 2014).

Several miRs, including miR-1, miR-21, miR-24, miR-26a, miR-29b, miR-143/145, miR-146a, miR-221/222, and miR-663, regulate dynamically VSMC differentiation and phenotypic switching in vitro, in animal experimental models and in human vascular pathologies (Davis-Dusenbery et al. 2011). miR-133a and miR-133b are expressed in VSMC at levels comparable to those of some typical vascular-enriched miRs (Albinsson et al. 2011; Torella et al. 2011).

The modulatory role of miR-133 in the phenotypic switch of cultured VSMCs has been described in recent reports (Torella et al. 2011; Liao et al. 2013; Gao et al. 2014). miR-133 appeared downregulated in proliferating VSMCs while it was upregulated when quiescence was induced in vitro. Proliferation of VSMCs was prevented by miR-133 overexpression and augmented by anti-miR-133 (Nazari-Jahantigh et al. 2012; Torella et al. 2011). Of note, the MAPK/ERK1/2 signaling pathway constitutes a key regulatory mechanism involved in miR-133 downregulation in cultured VSMCs primed for phenotypic switching. The

mechanism of the antiproliferative effect of miR-133 in vitro involves direct repression of its target, the specificity protein 1 or Sp1 (Torella et al. 2011), a transcription factor activated in VSMCs by phenotypic switch promoting stimuli, which is a known regulator of the Krüppel-like factor 4 (KLF4)/myocardin axis (Owens et al. 2004).

The antiproliferative effect of miR-133 observed in vitro was successfully translated to the experimental animal. Thus, proliferating VSMCs from rat carotid arteries subjected to angioplasty exhibited strongly reduced miR-133 levels in comparison with normal uninjured arteries (Ji et al. 2007; Torella et al. 2011). Arterial overexpression of miR-133 by adenoviral infection reduced VSMC activation and neointimal hyperplasia after the balloon injury. On the contrary, systemic administration of anti-miR-133 exacerbated VSMC hyperplasia which resulted in an increased neointimal formation, thickening of the tunica media, and restenosis (Torella et al. 2011). VSMCs from advanced atherosclerotic lesions of $ApoE^{-/-}$ mice expressed lower levels of miR-133a which associated downregulation of the insulin-like growth factor-1 receptors, and, as a result, VSMC proliferation was attenuated (Gao et al. 2014).

Contractile VSMCs may also experience a phenotypic transition into osteoblast-like cells, capable of synthesizing the proteins required for calcification, a hallmark feature of pathological arterial calcification. This entity is a complex, highly organized, and regulated process, similar to bone formation, which has been observed in cardiovascular diseases including atherosclerosis, Mönckeberg's medial calcific sclerosis, aortic valve calcification, and calciphylaxis (Demer and Tintut 2008). VSMC can transdifferentiate into osteo/chondrocytic-like cells by upregulation of transcription factors such as runt-related transcription factor 2 (Runx-2), a validated target of miR-133a. Studies in vitro have shown that β-glycerophosphate-induced osteogenic differentiation of VSMCs associated downregulation of miR-133a levels. Gain- and loss-of-function experiments, using miR-133a mimics and inhibitors, identified miR-133a as a negative regulator of osteogenic transdifferentiation of VSMCs by targeting Runx2. Accordingly, the pro-osteogenic effect of miR-133a inhibitor was abrogated in Runx2-knockdown cells, whereas overexpression of Runx2 prevented miR-133a-induced inhibition of VSMC transdifferentiation into osteoblast-like cells (Liao et al. 2013).

Several studies investigated in patients the contribution of miR-133 dysregulation to the pathological remodeling of the vascular wall in diseases such as essential hypertension and arterial aneurysms (Lacolley et al. 2012). VSMC-modulating miRs, including miR-133, have been reported to be closely related to essential hypertension in humans. Thus, in a cohort of patients with untreated essential hypertension, the expression levels of miR-133 in peripheral blood mononuclear cells correlated significantly and positively with clinical markers of the disease. Therefore, miR-133 might represent a useful biomarker of clinical status and potential therapeutic target in hypertension (Kontaraki et al. 2014).

Arterial aneurysm formation is a poorly understood, complex, multifactorial process of destructive vascular remodeling characterized by local chronic inflammation, oxidative stress, phenotypic switch of VSMC, VSMC loss, and

fragmentation of the extracellular matrix. Similar structural changes have been reported in intracranial (ICA), thoracic aortic (TAA), and abdominal aortic (AAA) aneurysms, though their etiology differs (Norman and Powell 2010). Arterial aneurysm rupture and dissection are major causes of morbimortality in the clinic, and there is still a need for accurate predicting aneurysmal growth and potential rupture risk (Norman and Powell 2010). The pathophysiological role of miRs in aneurysm formation and their value as biomarkers in the diagnosis, prognosis, and management of aneurysmal disease are a matter of intensive research (for reviews see Maegdefessel et al. 2013; Wei et al. 2013). In human patients, several studies comprehensively analyzed miR expression profiles for aneurysmal tissues of different locations. The profiles of regulated miRs may reflect the different genetic and molecular mechanisms involved in the etiology of the aneurysmal disease depending on location (Norman and Powell 2010). Most of these studies evidenced that miR-133a and miR-133b were consistently downregulated in both aortic and intracranial aneurysms (Table 3), which suggests that both miRs may be involved in some common pathways implicated in aneurysm formation.

Pahl et al. (2012), using miR microarray and qRT-PCR validation, reported downregulation of miR-133b and miR-133a expressions in AAA from patients undergoing elective open repair compared with autopsy or tissue bank samples (Pahl et al. 2012). In a miR microarray study, Kin et al. (2012) reported a trend toward lower expression levels of miR-133a in atherosclerotic AAA, although ascending thoracic aorta was used as control tissue, which is a limitation of the study (Kin et al. 2012). On the other hand, Cheuk and Cheng (2014) did not detect differences in the levels of miR-133 in VSMCs isolated of aneurysm samples from AAA patients in comparison to normal VSMCs from sex-matched organ donors.

In the thoracic aorta, the expression levels of miR-133a and miR-133b, determined by microarray and qRT-PCR assays, appeared significantly downregulated in the diseased wall from patients with thoracic aortic dissection (TAD) compared with normal thoracic aorta samples from age-matched donors. Using bioinformatic tools, the authors proposed type-V collagen (COL5A3) and laminin subunit beta-3 (LAMB3) as relevant predicted targets for these two miRs (Liao et al. 2011). Similarly, Ikonomidis' group (Jones et al. 2011) reported a significant reduction of miR-133a expression levels in aortic tissue of ascending TAAs from patients with tricuspid aortic valve (TAV) in comparison with control specimens of ascending aorta, as determined by microarray and qRT-PCR analysis. Moreover, a significant inverse relationship between the expression level of miR-133a and the aortic diameter ($r = 0.42$, $p < 0.05$, $n = 25$) was evidenced (Jones et al. 2011).

In parallel, two studies analyzing miR dysregulation in ICA tissue samples using microarray and qRT-PCR analysis reported reduced expression levels of miR-133a and miR-133b in aneurysmal samples versus the control vessels from matched patients (Jiang et al. 2013; Liu et al. 2014). Bioinformatic analyses allowed the authors the prediction of a subset of potential relevant target genes of miR-133a/miR-133b, with regulatory roles on programmed cell death, extracellular matrix organization, response to oxidative stress, TGF-β signaling pathway, endothelial and

Table 3 microRNA-133 in patients with peripheral arterial disease

Pathology	Cohorts	miR-133 regulation	Other major findings	References
Systemic hypertension	Untreated hypertensive patients ($n = 60$), healthy controls ($n = 29$)	↓miR-133 in PBMC	↓miR-143, -145 ↑miR-21,-1 miR-133 correlated directly with 24-h diastolic BP and with the dipping status	Kontaraki et al. (2014)
Abdominal aortic aneurysms (AAA)	AAA patients ($n = 41$), autopsy and biobank controls ($n = 12$)	↓miR-133a/b Microarray Infrarenal aorta	Bioinformatic prediction of target genes involved in AAA	Pahl et al. (2012)
	AAA patients ($n = 13$) and controls from aortic valve replacement ($n = 7$)	Trend ↓mir-133a Microarray AAAs: infrarenal aorta Control: ascending aorta	miRs: ↑AAA tissue, ↓plasma: endothelial (let-7f, miR-20a, -21, -27, -92a, -126, -221, and -222), inflammatory (miR-124a, -146a,- 155, and -223), and fibrosis-related (miR- 29b)	Kin et al. (2012)
Thoracic aortic dissections and aneurysms (TAA, TAD)	TAD patients ($n = 6$) and control donors ($n = 6$)	↓miR-133a/b Microarray, qRT-PCR TAA segments	↑18 miRs; ↓56 miRs Bioinformatic prediction of targets, networks and pathways	Liao et al. (2011)
	TAA ($n = 30$) and control donors or CABG patients ($n = 10$)	↓miR-133a Microarray, qRT-PCR TAA segments	miRs -1, -21,-29a, -133a, and -486 down- regulated Predicted targets: MMP-2 for miR-29a and MMP-9 for miR-133a	Jones et al. (2011)
	TAA patients with BAV ($n = 21$) or TAV ($n = 21$) and control donors/ receptors ($n = 10$)	↓miR-133a: TAA segments ↑miR-133a: Plasma Microarray, qRT-PCR	Dysregulated miR-1, -21, -29, -143, -145. Little concordance plasma versus tissue TAA associated with TAV or BAV: differential predictive models (ROC curves)	Ikonomidis et al. (2013)
Intracranial aneurysm (ICA)	Ruptured ICA ($n = 14$) and matched controls ($n = 14$)	↓mir-133a/b Microarray, qRT-PCR ICA tissue versus normal middle meningeal artery segments	↓18 miRs Bioinformatic prediction: targets, networks and pathways	Jiang et al. (2013)

(*continued*)

Table 3 (continued)

Pathology	Cohorts	miR-133 regulation	Other major findings	References
	ICA patients ($n = 6$) and matched controls ($n = 6$)	↓mir-133a/b Microarray, qRT-PCR ICA tissue versus normal superficial temporal artery segments	↓85 miRs, ↑72 miRs Bioinformatic prediction of targets, networks and pathways	Liu et al. (2014)
	ICA patients ($n = 18$) and controls ($n = 6$)	No change miR-133a/b qRT-PCR Serum	Bioinformatic prediction of targets, networks and pathways	Jin et al. (2013)
	ICA patients ($n = 40$) and healthy volunteers ($n = 20$)	No change miR-133a/b Microarray Plasma	miR-16 and miR-25: independent factors for ICA occurrence in logistic regression analysis	Li et al. (2014)

BAV bicuspid aortic valve, *PBMP* peripheral blood mononuclear cells, *ROC* receiver operating characteristic, *TAV* tricuspid aortic valve

VSMC proliferation and phenotypic switch, activation of the inflammatory response, and loss of cells in the vessel wall (Jiang et al. 2013; Liu et al. 2014).

With regard to differential profiles of miR-133 in peripheral blood which could help as biomarkers for predicting the likelihood of aneurysm occurrence and/or rupture, the results are variable depending on aneurysm location. Ikonomidis et al. (2013) detected increased circulating levels of miR-133a in plasma samples from patients with TAA associated to either TAV or bicuspid aortic valve (BAV) in comparison to control patients. Univariate logistic regression analysis indicated that circulating miR-133a expression alone was not predictive of aneurysm presence. However, multivariable analysis revealed that the combinations of miR-133a with miR-143 and MMP-8 or miR-133a with MMP-2, TIMP-2, miR-143, and miR-145 predicted with high degrees of specificity and sensitivity the presence of TAA disease overall and the presence of bicuspid aortopathy, respectively. These data support the concept that specific etiologic subtypes of ascending aortic aneurysm disease present specific biological plasma signatures and that miR-133a, together with other analytes, might help to predict the presence of either aneurysmal disease in general or particular subtypes of aneurysmal disease (Ikonomidis et al. 2013).

Contrary to the findings in patients with TAA, Li et al. (2014) reported, in a cohort of 40 ICA patients (20 unruptured and 20 ruptured) and 20 healthy volunteers, no differences in miR-133 plasma levels between patients with ICA and healthy volunteers nor between patients with ruptured or intact ICAs. Similarly, Jin et al. (2013) failed to identify changes in the expression levels of miR-133a/b in a

microarray study carried out using serum from patients with aneurysms with daughter aneurysms, aneurysm without daughter aneurysms, ruptured aneurysms, and angiography negative group.

Other Diseases or Conditions

miR-133a is involved in the interindividual response variability to the anti-vitamin K anticoagulant drugs. Vitamin K 2,3-epoxide reductase complex subunit 1 (VKORC1) is the main target of dicoumarinic anticoagulant drugs and is also a validated target of miR-133a (Pérez-Andreu et al. 2013). Further, VKORC1 is also involved in the correct vitamin K-dependent γ-carboxylation of matrix-Gla protein (MGP), a natural local inhibitor of vascular and other soft tissue calcification. Thus, an increased expression of miR-133a would result in reduced levels of VKORC1, increased susceptibility to dicoumarinics, and diminished γ-carboxylation of MGP with higher tendency to soft tissue calcification (Pérez-Andreu et al. 2013). However, the precise value of miR-133a as a biomarker of these two phenomena, namely, the individual susceptibility to dicoumarinic anticoagulation and the tendency to develop soft tissue mineralization, has not been delineated as yet and awaits further research.

Owing to its specific expression in muscle cells and its role in skeletal muscle conservation and regeneration, miR-133a has been included in the group of the so-called dystromirs. However, there is a paucity of data concerning the use of its circulating levels as a clinical marker of activity of skeletal myopathies. Plasma levels of miR-133a have been proposed as reliable indicators of experimental muscular damage in rats both by the use of toxics (Miwa et al. 2015) or in a model of Duchenne's muscular dystrophy (Roberts et al. 2012). In patients with myotonic dystrophy type 1, the most common type of muscular dystrophy in adults, circulating levels of miR-133a exhibit an excellent discriminant power for the diagnosis, reflect accurately the clinical stage of muscular impairment and correlate, inversely and significantly, with a standard muscle strength score (Perfetti et al. 2014). In children with Duchenne muscular dystrophy, miR-133a serum levels tell us about the remaining muscle mass of the patient, and there is a suggestion that might also help to monitor the therapeutic effect of dystrophin restoration treatments (Zaharieva et al. 2013).

Summary Points

- miR-133 is abundantly expressed in the heart and in vascular smooth muscle cells.
- Bioinformatic tools predicted 399 mRNA targets for miR-133a, and 42 have already been validated experimentally.
- miR-133 plays major roles in cell differentiation to cardiomyocytes during early development and in later stages of cardiac morphogenesis.

- miR-133 cooperates in experimental reprogramming of fibroblasts into cardiac-like myocytes.
- Dysregulation of miR-133 is involved in the pathological myocardial remodeling, in the transition to heart failure and its evolution.
- In LV hypertrophy induced by pressure overload, miR-133 decreases in both myocardium and plasma, and its levels predict LV reverse remodeling after overload release.
- In acute coronary events, miR-133 levels decrease in the ischemic myocardium and increase in the circulation proportionally to the extent of the infarcted area.
- The value of circulating miR-133 as a diagnostic and prognostic biomarker in patients with coronary artery disease has been established.
- Dysregulation of miR-133 contributes to the pathological vascular remodeling underlying essential hypertension, vascular calcification, atherosclerosis, and aneurysmal disease.
- Circulating miR-133a, together with other analytes, can help to predict the presence of either thoracic aneurysm in general or particular subtypes of aneurysmal disease.
- Circulating levels of miR-133 failed to predict the presence of intracranial aneurysms.

Acknowledgments This work was supported by the Ministerio de Economía y Competitividad, Spanish Government [Instituto de Salud Carlos III (PI12/00999 and RETICS RD12/0042/0018); SAF2013-47434-Retos].

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Troponin Elevation Beyond Coronary Arteries

14

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Contents

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_41

Abstract

Cardiac troponins are protein complexes that have become the gold standard biomarkers in the detection of myocardial injury. The use of new-generation high-sensitivity assays, which can detect even small increases in troponin levels, resulted in an increase in the number of patients with elevated troponin concentrations. However, in this case there are more false-positive results. This makes it of paramount importance to set differential diagnosis among several noncoronary entities such as stroke, pulmonary embolism (PE), sepsis, acute perimyocarditis, Takotsubo, acute heart failure (HF), and tachycardia. Technological progress of high-sensitivity troponin assays may be helpful in detecting even slight elevations of troponin in individuals, a condition that is met in several different clinical pathologies. However, despite the fact that troponin elevation is indicative of myocardial necrosis, it does not elucidate the pathophysiologic mechanism that causes myocardial damage. The purpose of this chapter is to report clinical pathologies where elevated troponin concentrations are found and to cite studies that have used troponin in the prediction and evaluation of future events.

Keywords

Troponin elevation • Sepsis • Stroke • Cardiomyopathies • End-stage renal disease

Abbreviations

ACC	American College of Cardiology
ACS	Acute coronary syndromes
AMI	Acute myocardial infraction
AV	Atrioventricular
CAD	Coronary artery disease
CKD	Chronic kidney disease
cTn	Cardiac troponin
ESC	European Society of Cardiology
ESRD	End-stage renal disease
HF	Heart failure
IL	Interleukin
MI	Myocardial infraction
MRI	Magnetic resonance imaging
PE	Pulmonary embolism
PSVT	Paroxysmal supraventricular tachycardia
SAH	Subarachnoid hemorrhage
SIRS	Systemic inflammatory response
SRCs	Stress-related cardiomyopathies
TAVI	Transcatheter aortic valve implantation
Tn	Troponin
TNF-α	Tumor necrosis factor-α
VARC	Valve Academic Research Consortium

Key Facts

- Troponin elevation occurs in patients with ESRD, tachyarrhythmias, acute HF, aortic stenosis, pericarditis and myocarditis, acute pulmonary embolism, stress-related cardiomyopathies, sepsis, stroke, strenuous exercise, and cardiac contusion apart from coronary events.
- Etiology of troponin elevation is multifactorial.
- Most of recently available data support the prognostic value of troponin on patients' outcome.

Definitions

Acute heart failure Acute heart failure is a cardiac pathology that is characterized by the inability of the heart to provide enough volume in order to satisfy the body's needs. Several entities are implicated with acute heart failure such as valvulopathies, coronary artery disease, and a damaged or inflamed heart.

End-stage renal disease End-stage renal disease is a clinical pathology that is characterized from declined function of kidneys. The function is not adequate for the everyday needs of the human body resulting to either hemodialysis or need for kidney transplantation. It is usually the subsequent consequence of chronic kidney disease, kidney injury/trauma, or major blood loss.

High-sensitivity troponin assay High-sensitivity troponin assays are troponin tests that have been designed to sense even slight elevations in concentrations in total population comparing with the conventional ones. The coefficient of variance (CV) of $<10\,\%$ at the 99th percentile value in the population of interest has been proposed by experts.

Subarachnoid hemorrhage Subarachnoid hemorrhage is the blood concentration in the subarachnoid space. This space is found among the brain and the thin tissues that cover the brain. It might be a result of several bleeding types (arteriovenous malformation, bleeding disorder, bleeding from a cerebral aneurysm, head injury).

TAVI Transcatheter aortic valve implantation constitutes an alternative treatment option for patients with severe symptomatic aortic stenosis who cannot undergo surgery due to the fact that they are either considered as "high risk" or inoperable. It can be performed via the femoral or subclavian artery or direct through the ascending aorta or transapical.

Introduction

Troponins are protein complexes that are composed of three subunits (troponins I (TnI), T (TnT), and C (TnC)). TnT binds to tropomyosin, TnC binds to calcium ions, and TnI binds to actin by preventing actin–myosin interaction (Antman 2002).

Troponins are specific for skeletal and cardiac muscle but not for smooth muscle. A percentage of 7 % of cardiac TnT (cTnT) and 3.5 % of cTnI is found in the myocyte cytoplasm of the heart. cTnT content per gram of myocardium is almost twofold higher than that of cTnI (Adams et al. 1993; Antman 2002). Besides, different genes produce cardiac and skeletal troponins in each type of muscle. However, the amino acid sequence of TnC is not different among two types of muscle. Therefore, its detection is not diagnostic (Schreier et al. 1990).

Both cTnI and cTnT are myocardial injury-specific markers. However, the used troponin assays differ significantly as far as sensitivity and specificity are concerned. Assays of cTnT that are industrially made by a single producer present with relatively uniform cut-off concentrations. However, first-generation troponin assays may have falsely detected skeletal muscle troponin as elevated cardiac troponin. In addition, cTnI assays, given the fact that different kits are used to detect different epitopes, differ concerning cut-off concentrations and standardizations (Ammann et al. 2003). As far as the upper reference limit of cTns is concerned, it was initially defined as the 97.5th percentile of the values measured in the normal control population (1). However, a later definition was that acute myocardial infarction (MI) is diagnosed when cTnI or cTnT concentrations, that are identified within 24 h after the initial event, are higher than the 99th percentile using a coefficient of variation of 10 % or less (Panteghini et al. 2004; Thygesen et al. 2012). However, values in the intermediate zone are indicative of minor myocardial damage (Ammann et al. 2003).

Cardiac troponins have raised to be the gold standard for the detection of myocardial injury (Thygesen et al. 2012) especially after the introduction of new generation, high-sensitivity assays in use that can detect even minor elevations in troponin concentrations (Giannitsis et al. 2010). The new high-sensitivity troponin methods give the opportunity to detect even minor damages on the cardiac heart muscle increasing the number of patients with elevated troponin concentrations. In this case, there is higher percentage of false-positive results. This makes it of paramount importance to differentiate the diagnosis among several non-coronary entities, especially when troponin levels are high (Fig. 1).

Aetiology of Troponin Elevations

Troponin Levels in Patients with End-Stage Renal Disease

Patients with chronic kidney disease (CKD) (particularly those with end-stage renal disease [ESRD]) have a greater frequency of persistently elevated cardiac troponin comparing to patients who do not have CKD. The controversial issue related to the troponin elevation, as mentioned above, is that this is not due to reduced renal clearance but due to myocardial injury (Wang and Lai 2008; Newby et al. 2012). Kidneys cannot easily clear large molecules such as troponin molecule, a fact that makes difficult troponin to be cleared from serum. Nevertheless, it has been proposed that the troponin molecule is fragmented into smaller parts that can be easily identified by the troponin assays and it may be cleared from kidneys. The mechanism

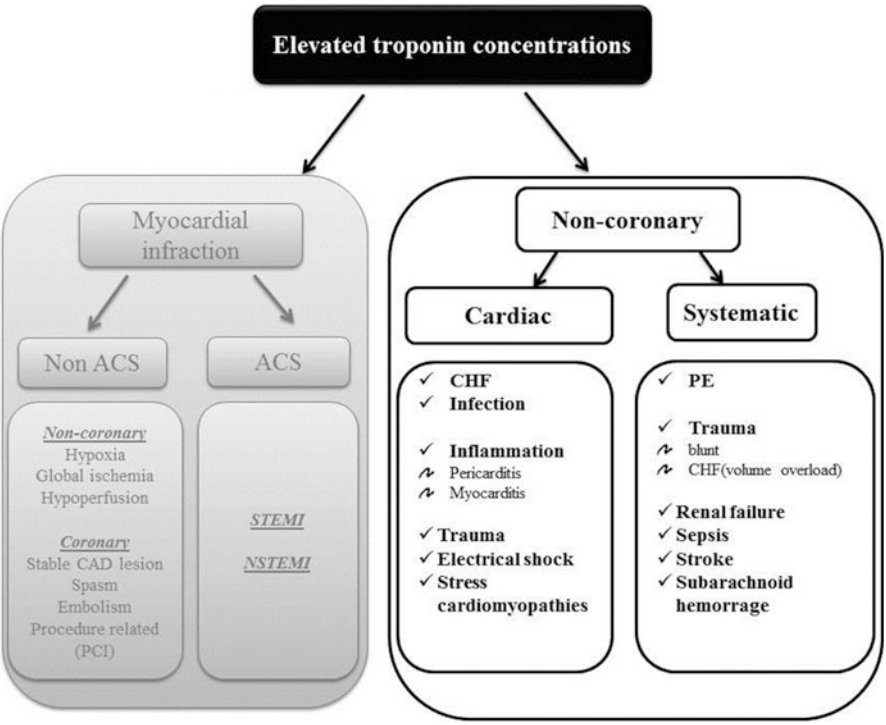

Fig. 1 Troponin elevation in coronary and non-coronary syndromes. Troponin concentrations are elevated in many pathological entities that they can be both coronary and non-coronary. The new high-sensitivity troponin methods give the opportunity to detect even minor damages on the cardiac heart muscle increasing the number of patients with elevated troponin concentrations. This makes it of paramount importance to set a differential diagnosis along than among several non-coronary entities

above may explain the elevation of troponin in severe renal failure (Diris et al. 2004). However, another study (Ellis et al. 2001) did not record a statistically significant difference among the half-life and the elimination rate of troponin I in patients with MI and ESRD when compared to those with MI and normal kidney function. Therefore, elevated troponin concentrations in patients with CKD should be assessed in the concept of acute coronary syndrome's (ACS) suspicion though they may also be due to other cardiac diseases associated with myocardial injury. This is highly prevalent among CKD patients, especially when the levels do not alter quickly over time (Jaffe 2006). In particular, in patients with CKD but without suspected ACS, micro-infarctions, microvascular disease, subendocardial ischemia associated with left ventricular hypertrophy and diastolic dysfunction, as well as non-ischemic cardiomyopathic processes could also be responsible for detectable small increases in troponin. Therefore, a change in the cTn concentration of more than 20 % has been considered as the main criterion for the diagnosis of MI in patients with ESRD with elevated cTn concentrations after symptoms' onset (Xu et al. 2013). This has

been considered an indicative change of three standard deviations (Wu et al. 2007). To be more specific, in a cohort of asymptomatic patients with ESRD, troponin levels exceeded the 99th percentile value using the new hs TnT assay in the entity of patients (Jacobs et al. 2009). Therefore, the wide variation in assays and thresholds along with the absence of comparative studies has not fully elucidated the relation among troponin concentrations and ischemia in patients with ESRD.

Tachyarrhythmias

Troponin elevation is commonly observed after episodes of tachyarrhythmias. However, the mechanism of tachycardia-induced troponin elevation is not fully understood (Ben Yedder et al. 2011). A widely proposed mechanism is that tachycardia increases myocardial oxygen demand with simultaneously decreased myocardial oxygen delivery, as a result of the short duration of diastole, which is the time when myocardial perfusion occurs. This results to reduced myocardial perfusion and release of cTnI (Carlberg et al. 2011). In animal studies, a second possible mechanism which is implicated with tachycardia-induced elevation of troponin concentrations proposes myocardial stretch. This is supported by the finding of a direct association with both a rise in natriuretic peptide and troponin concentrations (Qi et al. 2000). A probable scenario was that cTnI release from viable cardiomyocytes may be mediated by triggering stretch-responsive integrins. Their role is to link the extracellular matrix to the intracellular cytoskeleton (Hessel et al. 2008).

Paroxysmal supraventricular tachycardia (PSVT) is a commonly found arrhythmia. Usually, it is not dangerous; thus, it rarely leads to adverse clinical outcomes. However, a percentage of patients (30 %) with PSVT presented to have significantly elevated troponin concentrations (Ben Yedder et al. 2011). Furthermore, they presented with symptoms of chest pain and chest discomfort that were falsely diagnosed as acute ACS and consequently treated inappropriately with antiplatelet and antithrombotic therapies. Nevertheless, coronary angiography did not reveal serious pathology of coronary arteries in the majority of patients. Even more, it has been shown that patients with PSVT did not have risk factors that could increase cardiovascular risk (Ben Yedder et al. 2011). However, some predisposing factors that are closely related to troponin elevation, such as maximal PSVT heart rate, ST-segment depression $\geq$1 mm during the episode of PSVT, and impaired left ventricular systolic function, have been recorded (Chow et al. 2010; Ben Yedder et al. 2011).

Prolonged episodes of supraventricular tachyarrhythmias (SVT) have also been related with troponin elevation even in presumably healthy individuals. However, several limitations apply to these observations since no coronary angiography, stress testing, or hemodynamic measurements were performed in all patients, and continuous values of troponin changes were not available. In conclusion, whether tachycardia alone may cause a troponin release despite the absence of many cardiac entities (e.g., structural heart disease, significant CAD, and inflammatory mediators) or whether it is related to a disproportion between

oxygen demand and supply in patients with subclinical heart disease has not been fully elucidated yet.

Acute Heart Failure

Acute heart failure is a cardiac pathology where troponin elevation is rather common. However, the pathophysiologic substrate of troponin elevation in acute HF remains unclear. A possible scenario is based on the fact that increased ventricular preload triggers myocardial strain that may consequently result to troponin release (Feng et al. 2001). Another hypothesis is that the detectable elevated level of cTnT is due to myocardial damage as a result of necrotic and apoptotic processes. Thus, it has been estimated that 1 g of myocardial mass is being lost every year in the human heart (Olivetti et al. 1995). However, the present data have not fully clarified the question of whether the incidence of elevated troponin concentration and the width of increase/decline are significantly higher in acute comparing to chronic HF.

A number of studies have been realized in order to evaluate the possible relation among troponin elevation and adverse events. In particular, during the Acute Decompensated Heart Failure National Registry (ADHERE) Registry (Peacock et al. 2008), 67,924 HF patients were evaluated in order to discriminate the relationship among elevated troponin concentrations and adverse events. Out of them, 4,240 patients (6.2 %) had elevated troponin levels but using the less sensitive assays for cTnT or cTnI measurements. These patients when compared with those who did not have elevated troponin concentrations manifested lower values of systolic blood pressure and ejection fraction on admission and higher percentages of in-hospital mortality. Elevated troponin concentrations have been proved to be an independent predictor of mortality when adjusted for variables. These findings had previously been shown in another international pooled analysis of 1,256 acute destabilized HF patients (Januzzi et al. 2006).

Aortic Stenosis-Transcatheter Aortic Valve Implantation

Aortic stenosis constitutes a pathology that it is included in the most frequently encountered valvulopathies. Until recently, surgical valve replacement was the gold standard for the management of patients with symptomatic severe aortic stenosis. However, out of them, some patients are characterized either as “high operative risk” or “inoperable.” These patients are treated with transcatheter aortic valve implantation (TAVI), a technique that constitutes a well-established therapeutic alternative (Vavuranakis et al. 2010). In the setting of TAVI, myocardial biomarkers, like troponin, have also been incorporated in the current guidelines of the Valve Academic Research Consortium (VARC) for the detection of peri-procedural myocardial infarction (Leon et al. 2011). In some patients, TAVI is performed via transapical approach. This involves direct myocardial injury that may have increased troponin concentrations and consequently prognosis. Furthermore, the influence of

pre-interventional troponin levels on subsequent troponin release and outcome after TAVI has not been studied yet. Thus, the assessment of baseline values of cTn and its influence on outcome after TAVI are crucial to enlighten the role of troponins. In a study of 198 consecutive patients who underwent successful transfemoral TAVI, the relation of cTnT with procedural and 12-month outcome has been evaluated using a new-generation troponin T assay before and after TAVI. Furthermore, the relation of cTnT to the long-term outcome of the procedure has also been recorded. They showed that post-interventional cTnT levels increased significantly and peaked at day 3 after transfemoral TAVI, and they subsequently declined. Furthermore, they showed that baseline renal function, the duration of rapid ventricular pacing, as well as baseline cTnT values predicted the width of post-interventional cTnT concentrations. Despite the fact that cTnT levels did not prove to be an independent predictor of short-term mortality, pre-interventional as well as post-interventional cTnT concentrations predicted 1-year mortality, independently of procedural success (Chorianopoulos et al. 2014). In the first Department of Cardiology, a study that evaluated a total of 115 consecutive patients who were chosen for TAVI and were separated into groups according to post-procedural cTnI levels, was conducted. Patients with elevated TnI appear to have increased DQTc. DQTc was defined as that difference among the final and the pre-procedural value of QTc. It has been well recognized that QT prolongation is a marker of myocardial injury induced by ischemia, but in this study it probably represents myocardial necrosis induced both by ischemia or mechanical stress. Indeed, the presence of microembolization during balloon valvuloplasty and hypotension during rapid right ventricular pacing or even the use of medication that changes myocardial function during sedation/anesthesia may be implicated with myocardial necrosis even in the absence of significant epicardial coronary artery disease. Furthermore, new onset first-degree atrioventricular (AV) block appeared to be with higher. To conclude, the primary finding of this study was that TnI elevation after TAVI may be related to conduction abnormalities. The proposed mechanism for their appearance is probably associated with minor myocardial injury that affects the conduction system of the heart (Vavuranakis et al. 2013).

Pericarditis and Myocarditis

cTn has been recorded to be increased in 32–49 % of cases of acute pericarditis. Despite the fact that troponin is not present in the pericardium, probably this is due to the fact that the epicardium participates in the inflammatory process (Brandt et al. 2001).

Concerning the pathophysiologic mechanism of myopericarditis with sero-epidemiologic studies, data are limited. This suggests that most of patients with Coxsackie B virus infection are not detected and the inflammatory cascade is not widely expanded. Elevated troponin concentration is roughly related to inflammatory process but without adverse events in myopericarditis (Remes et al. 1990). Indeed, the finding above had been confirmed (Imazio et al. 2008); thus, acute

pericarditis and myopericarditis after 1 year had similar percentages of complications with echocardiography, ECG, and treadmill testing findings returning to normal patterns in most of cases. Nevertheless, the mechanism of myocarditis remains unclear, and cTn levels may have a significant range from normal levels up to high levels. To be more precise, primary myocarditis is supposed to be triggered either by acute viral infection or post-viral autoimmune response. This may of course predispose to coronary vasospasm too (Yilmaz et al. 2008). This partly justifies atypical chest pain in individuals with myocarditis that makes difficult the differential diagnosis with ischemic events. However, we should not underestimate the fact that MRI in endocarditis shows involvement from the epicardial layers, while ischemia is located in the endocardial layers extending toward the epicardial.

Acute Pulmonary Embolism

Elevated cardiac troponin levels in PE are present even in hemodynamically stable patients. The exact mechanism of troponin release in PE has not been fully elucidated yet. One explanation is that right ventricular strain that develops acutely due to increase in pulmonary artery resistance may be implicated for elevated troponin concentrations. Indeed, it has been recorded (Meyer et al. 2000) that a percentage of 63 % of patients with PE and right ventricular dilation manifested elevated cTnI concentrations, while 29 % of patients with positive cTnI test had a normal right ventricular end-diastolic diameter. Furthermore, an equally significant finding was that a positive cTnI level was associated with more segmental defects on ventilation–perfusion scintigraphy. Another scenario is that perfusion–ventilation mismatch induces hypoxemia that leads to hypoperfusion due to both impaired output and diminished coronary blood flow. In addition, the exploration of cTnT release mechanism in patients with PE indicated that peak values of the enzyme were lower comparing to those of individuals with acute MI and maintained high in blood for a shorter period (Muller-Bardorff et al. 2002). In particular, the mechanism of myocardial injury and cTnT release in patients with significant PE differs from the one in patients with ACS. In a meta-analysis (Becattini et al. 2007) of 20 studies in 1985 on patients with PE, it was found that increased cTn levels were adversely related with short-term mortality. They were also associated with a higher mortality in the subgroup of hemodynamically stable patients. Indeed, patients who were categorized as intermediate risk were hemodynamically stable but with right ventricular dysfunction or elevated troponins. This has been observed in a study where normal echocardiogram when combined with a negative cTnI was positively related with lower risk for early death (Kucher et al. 2003).

Stress-Related Cardiomyopathies

Stress-related cardiomyopathies (SRCs) are recorded as cardiac pathologies including Takotsubo cardiomyopathy or apical ballooning syndrome, subarachnoid

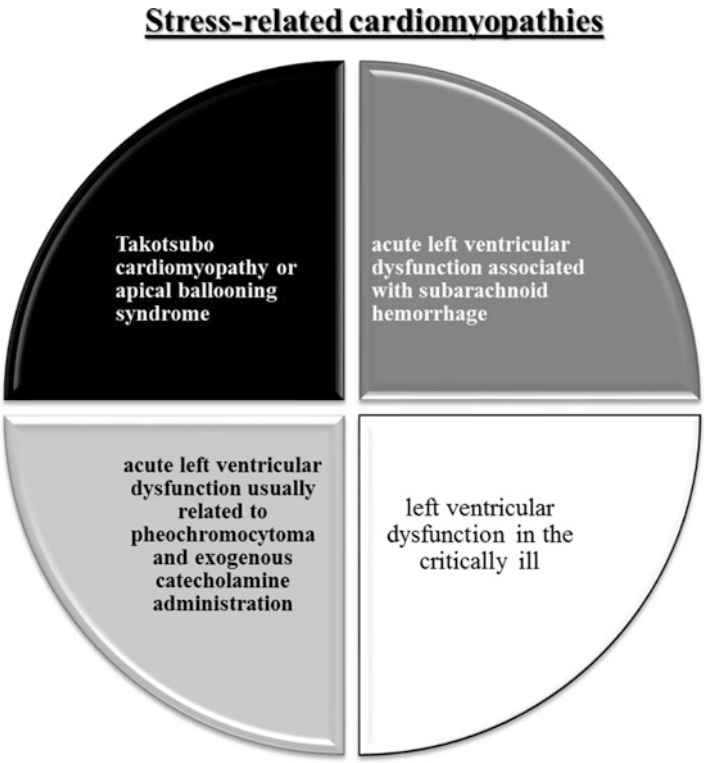

Fig. 2 Stress-related cardiomyopathies include Takotsubo cardiomyopathy or apical ballooning syndrome, acute left ventricular dysfunction associated with subarachnoid hemorrhage, acute left ventricular dysfunction usually related to pheochromocytoma and exogenous catecholamine administration, and acute left ventricular dysfunction in the critically ill

hemorrhage associated with acute left ventricular dysfunction, pheochromocytoma or critical state of patient, as well as exogenous catecholamine administration (Fig. 2). In these cases, cardiac toxicity mediated by catecholamines burdens left ventricular function and is accompanied by troponin release. Takotsubo cardiomyopathy has been characterized as a cardiomyopathy that is closely related with stress. Other characterizations for this syndrome include broken heart syndrome or transient left ventricular apical ballooning syndrome. Its prevalence is reported to range from 0.7 % to 2.5 % in patients presenting with acute coronary syndromes (Pilgrim and Wyss 2008). The typical Takotsubo cardiomyopathy syndrome includes women of older age with an acute emotional or physiologic stress (Fig. 3). Nevertheless, its clinical profile varies a lot and includes both younger patients and men (Sharkey et al. 2010). Emotionally or physically stressful events immediately before hospitalization are not always well defined in all patients with Takotsubo cardiomyopathy (Sharkey et al. 2010). The pathophysiology of syndrome has not been fully enlightened until recently; thus, several mechanisms have

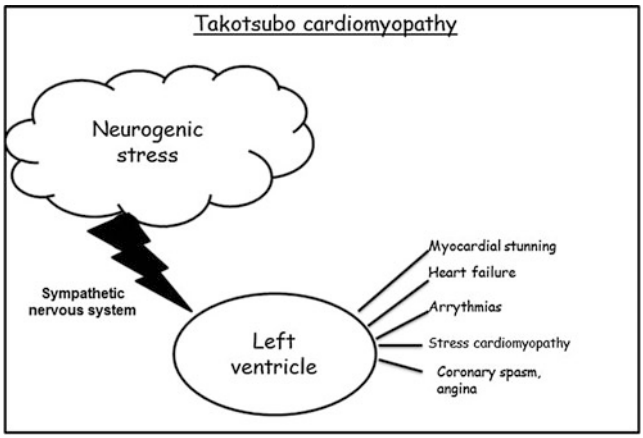

Fig. 3 The typical Takotsubo cardiomyopathy syndrome includes women of older age with an acute emotional or physiologic stress. Nevertheless, its clinical profile varies a lot and includes both younger patients and men

been suggested. Among them, catecholamine-induced myocardial stunning, ischemia-mediated stunning due to multi-vessel epicardial or microvascular spasm, aborted acute myocardial infarction (AMI), and focal myocarditis are included. The selective involvement of apical and/or midportion of the left ventricle with relative sparing of basal segments has not been fully clarified. Additionally, this might be partially enlightened by the fact that apical myocardium responds more to sympathetic stimulation. In these patients, symptoms including ischemic chest pain or dyspnea are usually present. The majority of patients with Takotsubo cardiomyopathy present a modest increase in cTn that reaches its peak within 24 h (Ramaraj et al. 2009). The paradox of this syndrome is that the elevation of ischemia biomarkers is lower for the degree of acutely induced regional wall motion abnormalities that appears acutely and remarkably lower than the one observed in ACS (Ramaraj et al. 2009). A prospective study was conducted in order to evaluate the extent of troponin T and I elevation in differentiating between Takotsubo cardiomyopathy and ACS. In that study, it was found that those with values of TnT over 6 ng/mL or with values of TnI over 15 ng/mL had smaller possibility to present Takotsubo cardiomyopathy (Sharkey et al. 2008). There is no specific therapy for Takotsubo cardiomyopathy except for supportive therapy. This leads to improvement of systolic dysfunction as well as regional wall motion abnormalities in a small period (Sharkey et al. 2010).

Acute LV dysfunction associated with subarachnoid hemorrhage is referred as neurocardiogenic stunning (Bybee and Prasad 2008). Its predictors are four and include severe neurologic injury, plasma troponin increase, brain natriuretic peptide elevation, and female gender (Tung et al. 2004). The diagnosis of neurogenic SRC is highly related to the onset of several, even fatal arrhythmias as well as an elevated risk of vasospasm of cerebral arteries. The prolongation of QT interval, the elevation

of ST segment, and T-wave inversion with symmetrical pattern are concomitant with an increase in cardiac troponin. They are recorded in approximately two thirds of patients with severe subarachnoid hemorrhage (Bybee and Prasad 2008). Unlike Takotusbo cardiomyopathy, the differential diagnosis between neurogenic SRC and acute MI is usually difficult. However, a slight elevation in cardiac troponin in combination with the onset of non-coronary distributed wall motion abnormalities favors the diagnosis of neurocardiogenic stunning.

Furthermore, acute LV dysfunction presents in an approximately 33–50 % of critically ill patients who are hospitalized. Its main characteristic is the onset of a global LV dysfunction. When dilated cardiomyopathy is excluded from differential diagnosis, the mechanisms of global LV dysfunction can be partially elucidated by a direct catecholamine myocardial toxicity in several situations. These include tachycardia-induced cardiomyopathy, hypertensive crisis, sepsis, multiorgan dysfunction, and post-cardiac arrest syndrome. In the entities above, a high prevalence of myocardial injury as determined by cTnI levels was recorded despite the absence of ACS on admission to the intensive care unit (Ammann et al. 2003; Quenot et al. 2005). Furthermore, it has been observed that this myocardial injury was an independent predictor of in-hospital mortality even when adjusted for co-variables (Quenot et al. 2005).

Sepsis

Half of patients with severe sepsis and septic shock may present declined ventricular function that is associated with elevated cTn concentrations (Mehta et al. 2004). Among patients with sepsis or systemic inflammatory response syndrome (SIRS) who were hospitalized in intensive care units, high levels of cTn have been noted in frequencies ranging from 12 % to 85 % (Lim et al. 2006). Several factors including the different underlying causes of sepsis, the variety of used troponin assays, and the different applied cut-off values for cTn may contribute to the wide range of incidence. Furthermore, this study has indicated that elevated cTn concentration is an independent predictor of mortality in sepsis patients (Lim Qushmaq et al. 2006). The high incidence of cTn elevations in septic patients raises the question of the mechanism that leads to troponin release (Figs. 4 and 5). A possible scenario is the one of global myocardial ischemia that results to the release of cTn from damaged myocardial cells due to oxygen supply–demand mismatch due to fever and tachycardia. This results in reduced oxygen supply of the myocardium as a result of systemic hypoxemia from respiratory failure, microcirculatory dysfunction, hypotension, and sometimes anemia. Except for ischemia, there are many other parameters that may result in myocardial injury in the substrate of septic shock. Troponins that are in small quantities in cytosol may leak through the myocardial membrane independently of any damage to myofibril (Turner et al. 1999). Furthermore, a possible mechanism includes the direct cardiac injury and myocytotoxic effect of endotoxins, cytokines (interleukins (IL) 1β, IL-6, and tumor necrosis factor (TNF)-α), nitric oxide and endotoxins (Ammann et al. 2001) as well as activation of caspase

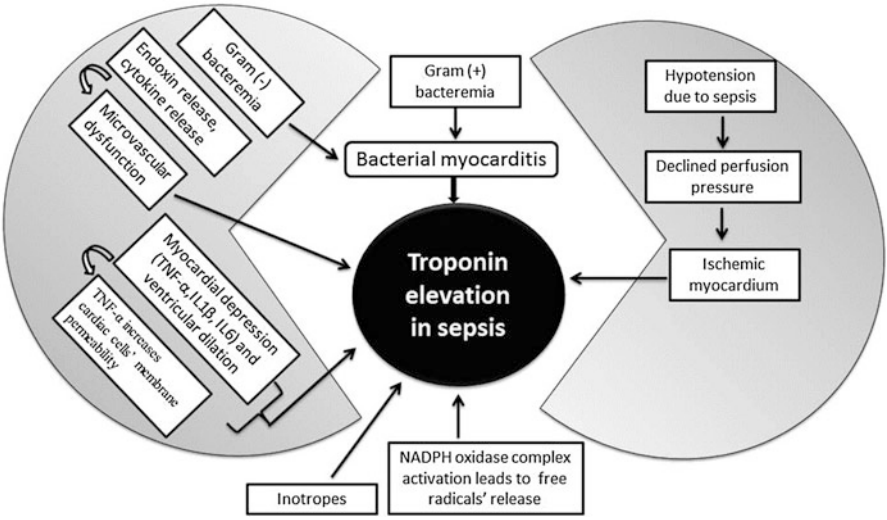

Fig. 4 The pathophysiologic mechanisms of sepsis. The high incidence of cTn elevations in septic patients raises the question for the mechanism that leads to troponin release. One of the possible mechanisms includes hypoxemia from respiratory failure, microcirculatory dysfunction, hypotension, and anemia. Another scenario is that small quantities of cytosolic troponins may leak though the myocardial membrane independently of any damage to myofibril. Myocytotoxic effect of endotoxins, cytokines (interleukins (IL) 1β, IL-6, and tumor necrosis factor (TNF)-α), nitric oxide, and endotoxins due to gram-negative bacteremia and sepsis results to myocardial depression and ventricular dysfunction. Another scenario includes the release of reactive oxygen radicals due to activation of NADPH oxidase complexes and mitochondria. These free radicals in combination with leucocyte-derived superoxide radicals are implicated with myocardial cell damage and apoptosis. Furthermore, a possible mechanism includes the direct cardiac injury. Finally, increased cardiac filling pressures and increased wall stress due to sepsis have been implicated with intracellular signaling cascade activation that leads to cardiac myocytes apoptosis, myocytes damage, and micronecrosis

3 (Communal et al. 2002) in case of gram-negative bacteremia and sepsis. Based on the fact that TNF-α increases the permeability of endothelial cells to macromolecules and lower molecular weight solutes, a similar increase in permeability of myocardial cell membrane could be expected (Brett et al. 1989). Additionally, IL1β, IL-6, and TNF-α have been proposed to play a central role in sepsis-mediated myocardial depression (Prabhu 2004). Indeed, in a recent study (Altmann et al. 2010), it has been shown that in a small group of patients with SIRS, sepsis, and septic shock, there were no differences among cTnI-positive and cTnI-negative patients when compared for coagulation parameters with thromboelastometry. They proposed that cytokines release from myocardial membrane, especially TNF-α, IL1β, and IL-6 play a crucial role in mediating hemodynamic effects and increase of cardiac troponin in patients with severe sepsis and septic shock. Another scenario includes the release of reactive oxygen radicals (Natanson et al. 1989) due to activation of NADPH oxidase complexes in mitochondria (Levy et al. 2005; Chagnon et al. 2006). These free

Fig. 5 Theories of cTn elevation in sepsis. Several theories have been proposed in order to elucidate troponin elevation in sepsis. These include: the demand and supply mismatch theory, the stress-mediated elevation of troponin during sepsis, the myocarditis and the role of cytokine vasopressor theory, the microthrombosis theory, and the ventricular wall stress-mediated theory

radicals in combination with leucocyte-derived superoxide radicals are implicated in myocardial cell damage and apoptosis (Levy et al. 2005). Finally, increased cardiac filling pressures and increased wall stress due to sepsis have been implicated in intracellular signaling cascade activation that leads to cardiac myocyte apoptosis (Horwich et al. 2003), myocyte damage, and micronecrosis (Brett et al. 1989). Whether cTn is indicative of reversible or irreversible myocardial damage remains unclear. However, in a recent meta-analysis (Sheyin et al. 2015) of 17 studies with total sample size of 1,857 patients, elevated troponin was proved to be an independent predictor of mortality (risk ratio, 1.91; 95 % CI, 1.65e2.22; $p < 0.05$).

Stroke

All types of stroke [ischemic, intracerebral hemorrhage, and subarachnoid hemorrhage (SAH)] are characterized by increased cTn levels (Sandhu et al. 2008). In particular, in a recent meta-analysis of 15 studies that involved 2,901 patients with acute stroke, a percentage of 18 % of them had elevated cTn concentrations with range from 0 % to 35 % probably due to different exclusion criteria and cTn cut-

offs (Kerr et al. 2009). The levels of cTn and adverse outcomes are closely related in the majority of studies that examine the relation among cTn and stroke (including SAH). In particular, in a recent meta-analysis (Kerr et al. 2009) on acute stroke patients with a positive troponin level, it seemed to express features representative of myocardial ischemia on the ECG and had poorer survival when compared with stroke patients without troponin elevation. Furthermore, several studies proved a strong positive correlation between cTn elevation and severity of the stroke (Ay et al. 2006). This constitutes cTn as a valuable biomarker for the evaluation of stroke severity despite the fact that the mechanism of increased cTn in the substrate of stroke has not been fully clarified. Undoubtedly, the extent of the ischemic plaque of the brain as well as the location of stroke influences the prognosis. Nevertheless, when patients survive after a stroke, other cardiovascular entities including coronary artery disease may affect the long-term survival (Dixit et al. 2000). cTn increase maybe also provoked by heart and renal failure rather than MI. cTn increase is also attributed to left ventricular systolic dysfunction which is encountered in all three kinds of strokes. Left ventricular dysfunction may be due to either exaggerated catecholamine release. This may also lead to a form of an unrestrained myocardial stress test that reveals ischemia by obstructive stable coronary plaques or can trigger Takotsubo disease.

Strenuous Exercise

Strenuous exercise may induce the release of cTn immediately after prolonged running (Scharhag et al. 2005; Sahlen et al. 2009). The proposed mechanism is that prolonged exercise causes muscular fatigue that is expressed as rapidly decreased systolic and diastolic function which is the so-called cardiac fatigue (Douglas et al. 1987). In particular, runners with elevated troponin concentrations after the race have also been proved to have more signs of right and left ventricular dysfunction including regional wall motion abnormalities (Neilan et al. 2006). In a meta-analysis, a percentage of 47 % of individuals had elevated troponin T after endurance exercise (Shave et al. 2007). Nevertheless, in another recent study using high-sensitivity troponin assays, the majority of marathon runners (80–86 %) had increased levels after racing (Mingels et al. 2009). In general, high-sensitivity troponin assays have indicated that even a short-duration exercise may result to elevated troponin concentrations if the intensity is high. To be more specific, it has been shown that a 30 min of high-force exercise led to small TnI elevations in 75 % of participant (Shave et al. 2010). However, others have proved that the troponin release is not necessarily indicative of myocardial injury. This is based on the fact that since the elevation usually normalizes within 24–48 h, at least in case of non-high-sensitive troponin assays (Scharhag et al. 2005). The proposed mechanism is that the released troponin is the product of degraded "cytosolic" troponin under stress. Undeniably, data from a murine model of forced physical stress support the above hypothesis (Chen et al. 2000). Fatigue symptoms are usually observed in long-distance runners. Therefore, the setting of troponin elevation combined with dizziness, chest pain, or collapse should constitute a challenging

Table 1 Mechanisms and characteristics of cTn elevation according to exercise intensity

Intense exercise
• Strenuous exercise may induce the release of cTn immediately especially after during prolonged running (Scharhag 2005, Sahlen 2009) • Runners with elevated troponin concentrations after the race have also been proved to have more signs of right and left ventricular dysfunction including regional wall motion abnormalities (Neilan et al. 2006) • 47 % of individuals had elevated troponin T after endurance exercise (Shave 2007) • The majority of marathon runners (80–86 %) had increased levels after racing (Mingels 2009)
Short duration exercise
• Short-duration exercise may result in elevated troponin concentrations if the intensity is high • It has been shown that 30 min of high-force exercise led to small TnI elevations in 75 % of participants while others have proved that the troponin release is not necessarily indicative of myocardial injury (Shave 2010) • The elevation usually normalizes within 24–48 h, at least in case of non-high sensitive troponin assays (Scharhag et al. 2005) • The proposed mechanism is that the released troponin is the product of degraded "cytosolic" troponin or prolonged staying of the myocytes' cell membranes under stress

diagnostic issue (Shave et al. 2005). The mechanisms of cTn elevation according to exercise intensity are summarized in Table 1.

Cardiac Contusion

Cardiac contusion that is a frequent enough entity is induced by blunt trauma on the chest wall. From the data of the literature, the frequency ranges from 5 % to 50 % with traffic accidents being one of the most usual reasons of cardiac contusion as a result of violent fall, aggressive impacts, and the practice of risky sports (Fabian et al. 1988). The range of post-traumatic cardiac lesions varies from no symptoms to decrease in cardiac function. The early diagnosis of cardiac contusion is achieved with continuous electrocardiographic monitoring, serial electrocardiograms, echocardiography, and measurement of serum biochemical cardiac markers such as troponin as well as radionuclide imaging and coronary angiography. However, significant complications had been recorded in patients with blunt chest trauma in whom ECG findings were normal and serial assessment of cTn was within reference intervals (Schultz and Trunkey 2004). Furthermore, cTnI and cTnT were compared with less-specific biomarkers for superiority in the detection of cardiac damage due to myocardial contusion in patients with blunt chest trauma and hemodynamic stability. Furthermore, it was investigated whether they were associated with significantly worse long-term prognosis (Bertinchant et al. 2000). It has been shown that despite improved specificity of cTnI and cTnT, the main problem with the use of these biomarkers was the low-sensitivity as well as low predictive values in diagnosing myocardial contusion (Bertinchant et al. 2000). Levels of cTnI were further evaluated in children with thoracic non-accidental trauma. It has been shown that the elevation of cTnI level could be indicative of sufficient chest trauma and

independent of the presence of cardiac decompensation or shock from other causes (Bennett et al. 2011).

Potential Applications to Prognosis and Other Diseases or Conditions

Troponins T and I are perfectly appropriate for the detection and prediction of myocardial injury because they are cardiac-specific proteins. Detection of a rise and/or fall of the cTn levels is crucial for the diagnosis of acute MI (Jaffe 2006); thus, increased cTn levels are defined as a value exceeding the 99th percentile of a normal reference population and must be determined for each specific assay with appropriate quality control in each laboratory (Apple et al. 2007). The criteria for cTn elevated values are assay dependent including high-sensitivity assays. Nevertheless, they can be defined from the precision profile of each assay (Thygesen et al. 2010). These biomarkers reach their peak values shortly after MI and maintain them for a prolonged time. In large reperfused MI, typically the biphasic time-release pattern of cTn, as described above, is usual (Thygesen et al. 2010). The early appearing peak may inform for the quality of microvascular reperfusion, while the levels of cTn on day 3 or 4 are indicative of myocardial infarct size (Giannitsis et al. 2008). It is strongly proposed that troponin is released from cardiac myocyte cell immediately after the membrane is disrupted as a result of myocardial cell death (Fishbein et al. 2003). However, the fact that troponin is elevated during marathon running (Giannitsis et al. 2009) doubts the scenario that it is released only due to irreversible damage. Finally, cTn can be useful in detection of myocardial injury during intervention for structural heart diseases. However, their significance concerning the prognostic value of adverse events have not been thoroughly evaluated yet.

Conclusion

Troponin is considered to be a very powerful diagnostic tool that helps the differential diagnosis of acute coronary syndromes from other entities. Despite the fact that it is cell specific for the cardiac muscle, troponin is observed to be high in conditions that are not included in ACS. High-sensitivity troponin assays appear to have apart from strengths and unique characteristics, some limitations that can cause problems into clinical practice. The technological progress of high-sensitivity troponin assays may be helpful for their widespread use with high potential to detect even slight elevations of troponin in healthy individuals that is met in several different clinical pathologies. However, despite the fact that troponin elevation is indicative of myocardial necrosis, it does not elucidate the pathophysiologic mechanism that causes myocardial damage. Therefore, the need for ameliorating the tests used as well as for discovering more specific biomarkers for the differential diagnosis of clinical entities is mandatory.

Summary Points

- This chapter focuses on troponin elevation beyond coronary artery disease.
- Troponins are protein complexes that are composed of three subunits (TnI, TnT, and TnC).
- Cardiac troponins are the gold standard biomarkers in the detection of myocardial injury.
- The use of new high-sensitivity assays detects even small increases in troponin levels increasing the number of patients who are detected with elevated troponin concentrations.
- Troponin concentrations are elevated at several noncoronary entities such as stroke, pulmonary embolism, sepsis, acute perimyocarditis, Takotsubo, acute heart failure, tachycardia, and cardiac contusion.
- Patients with chronic kidney disease have a greater frequency of persistently elevated cardiac troponin probably because troponin molecule is too large for the kidneys to be cleared from serum.
- The most predominant scenario for troponin elevation in episodes of tachyarrhythmias is the imbalance between oxygen demand and supply to the myocardium when myocardial perfusion occurs.
- The pathophysiologic substrate of troponin elevation in acute HF is based either on the fact that increased ventricular preload triggers myocardial strain that may consequently result to troponin release or due to myocardial damage as a result of necrotic and apoptotic processes.
- Elevated cardiac troponin levels in PE are present even in hemodynamically stable patients. The proposed scenarios for the pathophysiologic mechanism are two. The first is based on the fact that increased pulmonary artery resistance results to acute right ventricular strains and to elevated troponin concentrations. The second one is that hypoxemia leads to increased troponin levels. Patients with acute PE and elevated troponin had worse outcome than those without.
- In stress-related cardiomyopathies, cardiac toxicity mediated by catecholamines burdens left ventricular function and is accompanied by troponin elevation.
- Patients with severe sepsis and septic shock usually present with declined ventricular function which is related with elevated cTn concentrations. There are several proposed mechanisms for the pathophysiologic cascade.
- All types of stroke [ischemic, intracerebral hemorrhage, and subarachnoid hemorrhage (SAH)] are characterized by increased cTn levels.
- Strenuous exercise may induce the release of cTn immediately, especially after prolonged running.
- Cardiac contusion following blunt chest trauma is not rare and ranges from no symptoms to decrease in cardiac function with cardiogenic shock being a rarely encountered manifestation. The diagnosis is set with continuous electrocardiographic monitoring, serial electrocardiograms, echocardiography, and measurement of serum biochemical cardiac markers such as troponin as well as radionuclide imaging and coronary angiography.

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Circulating Vascular Endothelial Growth Factor-1 in Cardiovascular Disease

15

Alexander E. Berezin

Contents

Abstract

Vascular endothelial growth factor-1 (VEGF-1) is a heterodimer with a glycoprotein structure that belongs to the superfamily of vascular endothelial growth factors with pronounced angiopoetic capacity in vivo. VEGF-1 is expressed in tissue due to hypoxia and inflammation by wide spectrum of the cells and contributes angiogenesis and neovascularization by several mechanisms. A paracrine regulation of the VEGF-1 activity is mediated by a specific solubilized receptor that plays a key role in a reduction of ischemic tissue injury by inducing target organ protection, neurogenesis, and angiogenesis. The clinical correlations of circulating levels of VEGF-1 in subjects with cardiovascular diseases are largely unclear. It has been suggested that exaggerated VEGF-1 level would confer a better

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_2

prognosis in CAD patients, while a negative effect of neovascularization in plaque region supported by VEGF-1 is defined. Therefore, the negative effect of VEGF-1 on progression in age-related diseases, such as early diabetic retinopathy, has been reported. This chapter is dedicated to the discussion of the controversial role of the VEGF-1 among patients with cardiovascular disease and an assay to predictive value of VEGF-1 as biomarker at risk stratification.

Keywords

Vascular endothelial growth factor • Angiogenesis • Neovascularization • Cardiovascular diseases • Age-related diseases • Metabolic comorbidities

Abbreviations

ACS	Acute coronary syndrome
CABG	Coronary artery bypass grafting
HIF-1α	Hypoxia-inducible factor-1α
MACE	Major adverse cardiac events
MI	Myocardial infarction
PCI	Percutaneous coronary intervention
PHD	Prolyl hydroxylase domain-containing protein
ROCK	Rho kinase
RVR	Renal vascular resistance
VEGF	Vascular endothelial growth factor
VEGFR	Receptor for vascular endothelial growth factor

Key Facts

See Tables 1, 2, and 3.

Table 1 Key facts of the biological effects of VEGF-1. This table lists the key facts of direct and indirect effects of VEGF-1

Direct effects	Indirect effects
Supporting of angiogenesis	Neuroprotection
Regulation of endothelial cell differentiation	Cardioprotection
Formation of vascular beds of several organs during embryogenesis	Nephroprotection
Stimulation of maturation of different origin progenitor cells	Anti-inflammatory capacity probably due to a nitric oxide-dependent manner
Contribution in endothelial cell nitric oxide production	Decreasing mononuclears adhesion

VEGF vascular endothelial growth factor

Table 2 Key facts of dual role of VEGF-1 in cardiovascular diseases. This table lists the key facts of dual role of VEGF-1 in cardiovascular diseases

Positive effects	Negative effects
Target organ protection followed by a potentially pathogenic induction of vascular remodeling	Neovascularization of plaque region with increased vulnerability of patients
Attenuation of endothelial lesion	Stimulation of instability of plaque
Support of cardiac pump and diastolic function	Increased tissue and vascular remodeling
Stimulation of collateral blood flow	Proapoptotic effect in nitric oxide dependent manner
Increase of vasa permeability	
Regulation of systemic blood pressure	
Stimulation of natriuresis	
Low-intensive anti-inflammatory effect	

VEGF vascular endothelial growth factor

Table 3 Key facts of predictive value of VEGF-1 in cardiovascular diseases. This table lists the key facts of episodical assessment or serial measurements of VEGF-1 level in cardiovascular diseases

Predictive value of VEGF-1 is possible	Predictive value of VEGF-1 is not clear
Acute MI, acute coronary syndrome	Pulmonary arterial hypertension
Urgent or postponed PCI or CABG	Anti-VEGF-1-induced hypertension
Acute myocarditis	Asymptomatic atherosclerosis
Acute or acutely decompensated chronic HF	Peripheral artery disease
Dilated cardiomyopathy with reduced LVEF	Systemic hypertension
Eclampsia and preeclampsia	Pulmonary thromboembolism
Atherothrombosis (?)	Diabetes mellitus
Ischemic stroke	Obesity
	Resistance to insulin
	Dyslipidemia

VEGF vascular endothelial growth factor, *MI* myocardial infarction, *ACS* acute coronary syndrome, *HF* heart failure, *PCI* percutaneous coronary intervention, *CABG* coronary artery bypass grafting, *LVEF* left ventricular ejection fraction

Definitions

Angiogenesis is considered a predominant form of neovascularization mediated by endothelial cells sprouting from postcapillary vessels, leading primarily to new vessel formation.

Culprit coronary artery lesions are defined as overall plaque burden and necrotic core usually with intraplaque hemorrhage or thrombosis

MicroRNAs are defined as a class of noncoding RNAs that play pivotal roles in the postprocessing regulation of gene expression and are involved in a wide range of biological processes such as cell cycle control, apoptosis, stem cell differentiation, hematopoiesis, hypoxia, cardiac and skeletal muscle development, neurogenesis, insulin secretion, cholesterol metabolism, aging, immune responses, etc.

Major adverse cardiac events is a term that accumulates nonfatal myocardial infarction and sudden cardiac death

Non-fibroatheroma is defined as phenotype associated with pathological intimal thickening or fibrotic and/or fibrocalcific lesions of plaque with high risk of rupture.

Neovascularization is defined as a process of generating new blood vessels mediated primarily by progenitor cells of different origin, including endothelial progenitor cells, leading to tube formation, resulting in a stabilized neovascular channel.

Vulnerable plaque is defined as all types of atherosclerotic plaques with high likelihood of thrombotic complications and rapid progression.

Vulnerable patient is a term that may be more appropriate than vulnerable plaque and is proposed for the identification of subjects with high likelihood of developing cardiac events in the near future

Introduction

Angiogenesis and neovascularization might have a controversial role in pathogenesis of several cardiovascular diseases. On the one hand, generating new blood vessels mediated is considered a powerful mechanism that leads to attenuation of ischemic damage and restoration of tissue perfusion. On the other hand, new vessel formation plays a critical role in the progression of atherosclerotic lesions and appearance of vulnerability. It is known that neovascularization may distribute to the plaque throughout the vessel wall resulting to induction of instability, mechanical disorders of the plaque cap, and rupture. Vascular endothelial growth factor-1 (VEGF-1) is a heterodimer with a glycoprotein structure. It belongs to the superfamily of vascular endothelial growth factors that are synthesized by a wide spectrum of cells and possessed a pronounced angiopoetic capacity in vivo. VEGF-1 is key player in the processing of revascularization neoangiogenesis, reperfusion, as well as neuroprotection and cardioprotection. This chapter is dedicated to the discussion of the controversial role of the VEGF-1 among patients with cardiovascular disease and an assay to predictive value of VEGF-1 as biomarker at risk stratification.

Biological Role of VEGF-1

Vascular endothelial growth factor-1 (VEGF-1) is a heterodimer with a glycoprotein structure and belongs to the superfamily of vascular endothelial growth factors that is synthesized by a wide spectrum of cells, and it possesses a pronounced angiopoetic capacity in vivo (Ferrara et al. 2003). By now it is known that VEGF-1 appears to be stimulated angiogenesis in several settings by signaling through VEGF receptor-2 (VEGFR-2, also known as FLK1) (Howangyin and Silvestre 2014; Holmes et al. 2007). VEGF is mainly expressed on the surface of wide spectrum of cells in various organs including placental syncytiotrophoblast cells and invasive chorionic trophoblast cells during pregnancy. VEGF-1 has its biological effect through cooperation with the tyrosine kinase receptors located on the endothelial cells' surface, which causes cell growth, proliferation, and migration, as well as neovascularization and angiogenesis (Takahashi and Shibuya 2005; Shen et al. 2011). VEGF-1, being a ligand for alpha-5/beta-1 integrin, was found to be able to activate the migration of mononuclears and endothelial cells, to potentiate vasodilation, and to increase an inflammatory response (Luque et al. 2003; Orecchia et al. 2003). A paracrine regulation of the VEGF-1 activity is mediated by a specific solubilized receptor that plays a key role in reduction of ischemic tissue injury by inducing target organ protection, neurogenesis, and angiogenesis (Siow and Churchman 2007). The key facts regarding VEGF-1 effects are in Table 1.

It is well-known that the initial stimuli for overexpression of VEGF-1 are active forms of oxygen in the tissue that may also modulate the expression of hypoxia-inducible factor-1α (HIF-1α) (Carmeliet and Jain 2011). Both growth factors are controlled by prolyl hydroxylase domain-containing proteins (PHD), which is considered a potential cardioprotective and neuroprotective factor, as well as a certain angiopoetic modulator (Reischl et al. 2014). As known, VEGF-1 increases the permeability of the layer of endothelial cells, leads to plasma proteins to extravasate and lay down a provisional extracellular matrix scaffold, and, thereby, promotes sufficient proangiogenic effect (Carmeliet and Jain 2011). Therefore, it is suggested that the glycoprotein 130- glycoprotein 130 ligand system may also be involved in VEGF-related regulation in human cardiac myocytes (Weiss et al. 2003). Indeed, increased VEGF-1 expression was found in myocardial tissue obtained from a patient with acute myocarditis, and a selective stimulation of VEGF by gp130 ligands was also reflected by a specific receptor expression on cardiac myocytes (Weiss et al. 2003). Because glycoprotein 130 is a common receptor subunit for several inflammatory cytokines, such as interleukins (IL) -6, IL-11, cardiotrophin-1, etc., the ability of glycoprotein 130- glycoprotein 130 ligand system to upregulate VEGF expression in the myocardium is crucial for maintenance of cardiac function in myocarditis and ischemic cardiomyopathies (Weiss et al. 2003).

In fact, several pathological processes, such as hypoxia and inflammation via induction of VEGF through auto- and paracrine mechanisms, may play a pivotal role in myocardial revascularization. Exaggerated production of VEGF-1 may depend on

overexpression of microRNAs (miRNAs) involved in the modulation of various angiopoetic factors. It has been found that miR-181a, miR-106a, and miR-20b are involved in biological processes associated with angiogenesis, such as the cell cycle, cell migration, cell growth, and proliferation, through modulation of VEGF-1 overexpression (Cuevas et al. 2014). VEGF-1 is able to improve survival of endothelial cells through an activation of intracellular regulating enzymes, such as PI3-kinase, Akt, and Src (Tsurumi et al. 1997). Overall, VEGF-1 promotes proliferative changes by two ways: a classical promotion of endothelial cell layer and a noncanonical ability to engage platelet-derived growth factor receptor α and gp130-gp130 ligand system (Pennock et al. 2014). We do not know whether one of these mechanisms is key player in the cardiac remodeling in patients with various settings and what is the predictive role of circulating VEGF-1 in different clinical settings.

VEGF-1 in Atherosclerosis and Coronary Artery Disease

The clinical correlations of circulating levels of VEGF-1 in asymptomatic atherosclerosis and symptomatic coronary artery disease (CAD), including unstable CAD subjects who are required PCI, are largely unclear. For acute and stable CAD, asymptomatic atherosclerosis and planned or postpounded revascularization procedures (CABG, PCI), VEGF-1 may produce multi-directed effects (D'Amario et al. 2014). Key facts of the dual role of VEGF-1 in cardiovascular diseases are in Table 2.

It is well-known that formation of new vessels from vasa origin characterized severely stenotic lesions and also correlated well with the extent of inflammatory cell infiltration of lipid core and lipid core size. VEGF-1 produced by peripheral blood mononuclear cells, which are accumulated in the plaque rupture . Interestingly, new vessels from lumen origin were found in plaques with 40 % and 50 % artery stenosis and were associated frequently with hemorrhage in the plaque (Kumamoto et al. 1995). On the one hand, there is close interrelationship between neovascularization and risk of plaque instability that is considered a potentially unfavorable condition for survival of the patients. On the other hand, the extent of ischemic myocardial damage and appearance of acute myocardial infarction (MI) contribute to the elevation of serum VEGF-1 levels that allows VEGF-1 to improve left ventricular function by promoting angiogenesis and reendothelialization after MI (Hojo et al. 2000). Indeed, there are evidences that the patients with acute MI have elevated circulating VEGF-1 levels when compared with healthy subjects (Seko et al. 1997). After reperfusion, the serum VEGF-1 levels rapidly returned almost completely to the normal control range. These data allowed authors to strongly suggest that the serum level of VEGF-1 is one of the most sensitive indicators of myocardial ischemia. Kranz et al. (2000) measured the levels of VEGF-1 in the serum and in the coronary sinus of patients after acute MI. Surprisingly, according to data obtained, the main source for VEGF-1 in the blood stream is not the infarcted myocardium, while concentration of VEGF-1 in coronary sinus was higher

compared with peripheral blood stream. Authors concluded that the most likely source of the elevated VEGF-1 in acute MI patients is circulating platelets, rather than the infarcted myocardium. However, obtained data of the investigation have suggested that VEGF-1 is key player in endogenous activation of coronary collateral formation in the human heart. This suggestion confirmed the results obtained by Ramos et al. (2014). Authors examined the longitudinal changes of VEGF-1 levels after PCI for predicting major adverse cardiac events (MACE) in CAD patients. The VEGF-1 concentration showed a positive evolution through 1 year in 84 % of patients enrolled in the study. The longitudinal changes of circulating VEGF-1 levels in the patients significantly increased to 1 month and remained relatively steady to 1 year approaching the VEGF-1 levels of healthy volunteers. Low baseline VEGF concentration (<40.8 pg/mL) conveyed increased risk for recurrent hospitalization and MACE in a 5-year follow-up after PCI with drug-eluting stent placement. According to opinion of investigators, the results reflect a positive role of elevated VEGF-1 in serum in recovery and support its importance in CAD prognosis. It is needed to take into consideration that VEGF-1 levels were below detection limit in almost 50 % of the acute MI or acute coronary syndrome (ACS) and non-ACS patients at the baseline in the majority of investigations dedicated to this issue. Notedly, the data obtained from patients with acute MI, who were not candidates for PCI, also indicated a sufficient predictive role of circulating VEGF-1(Korybalska et al. 2011; Heeschen et al. 2003). However, exaggerated VEGF-1 level would confer a better prognosis in CAD patients undergoing PCI or without it as its actions may contribute to ameliorate the damaged endothelium and promote rapid recovery after stenting and reperfusion due to thrombolysis.

Nevertheless, this issue seems to be not obvious, because there are evidences for negative effects of VEGF-1 toward atherothrombosis. There are at least two facts that confirmed a negative effect of neovascularization in plaque region (Subbotin 2012). The recent human researches have shown reducing microvessel formation in fibrocalcific plaques when compared with vulnerable, ruptured, and lipid-rich plaques that are considered a life-threatening find (Hansson 2005). Therefore, the second fact relating neovessels to plaque regression is the impressive 85 % and 70 % reduction of atherosclerosis in apoE-knockout mice treated with the angiogenic inhibitors endostatin and TNP-470, respectively (Moreno et al. 2006). Moreover, overexpression of VEGF-1 in endothelial cells and circulating mononuclears may contribute in thrombosis and thrombus remodeling (Hansson 2005). Because VEGF-1 is considered a key proangiogenic factor in atherosclerotic plaques, which is expressed in the necrotic nucleus of the atheroma (Cuevas et al. 2014), the final result of the expression will define plaque evolution and small vessel growth around an ischemic or necrotic zone (Al-Rasheed et al. 2013).

Thus, the role of the VEGF-1 in CAD patients may depend on clinical settings, requirement of reperfusion procedures, and, probably, type and generation of the stent. This issue requires more detailed investigations with higher statistical power, while preliminary reports regarding predictive role of exaggerated VEGF-1 levels seems to be optimistic.

The Role of VEGF-1 in Age-Related Diseases

The role of VEGF-1 in age-related diseases is still under discussion and appears to be very controversial. The negative effect of VEGF-1 on disease progression in age-related diseases, such as early diabetic retinopathy, has been defined very well in animal models and in the clinical studies (Yan and Su 2014; Abu El-Asrar et al. 2013). Overall, for diabetic patients with retinopathy, nephropathy, and neuropathy, the final result of stimulation of angiogenesis is certainly negative, on the other hand, for subjects with obesity, multiple sclerosis, amyotrophic lateral sclerosis, and Alzheimer's disease (Dejda et al. 2014; Taiana et al. 2014; Holmes et al. 2007). Probably the role of VEGF-1 overexpression is defined uncertain (Taiana et al. 2014; Tufro and Veron 2012). In fact, suppression of angiogenesis might be favorable in diabetic population (Mitry et al. 2013; Nicholson and Schachat 2010). Indeed, using anti-VEGF drugs has been shown that there is a positive response affecting metabolic faces of age-related diseases (Badros et al. 2005). However, these suggestions are not obvious (Schratzberger et al. 2001). Surprisingly, anti-VEGF treatment increased insulin sensitivity in young and old mice but had no effects in the mid-aged group. Therefore, anti-VEGF remedies significantly improved insulin sensitivity in mid-aged obese mice fed with a high-fat diet (Honek et al. 2014).

The innate exact mechanisms affected VEGF and their role in metabolism is still not understood. Because levels of VEGF expression in various white adipose tissues may change uninterruptedly in various age populations, it has been suggested that adipose vasculature sufficiently modulates fat mass, adipocyte functions, blood lipid composition, as wells as insulin sensitivity (Honek et al. 2014). By now it is known that the proangiogenic mononuclear phagocytes are able selectively recruited to sites of pathological neovascularization in response to locally produced semaphorin 3A as well as VEGF-1, that is, essential for disease progression (Dejda et al. 2014). Therefore, hyperglycemia may increase VEGF-1 and VEGFR mRNA without changing their intracellular protein levels in neurons of different origin, such as dorsal root ganglion that may lead to early affected neurite outgrowth through the impairment of VEGF/VEGFR signaling (Kennedy and Zochodne 2005; Leinninger et al. 2004; Zochodne et al. 2001).

Thus, VEGF-1-related pathological neovascularization is defined as a pivotal mechanism of negative evolution of age-related diseases, such as diabetes, obesity, and insulin resistance (Honek et al. 2014). More evidences for predictive role of VEGF-1 in diabetes and other metabolic comorbidities in patients with cardiovascular diseases are required. Recent clinical studies have shown that serum VEGF increases in diabetic polyneuropathy, particularly in the neurologically active symptomatic stage (Deguchi et al. 2009; Mironidou-Tzouveleki et al. 2011).

Anti-VEGF-1-Induced Hypertension

By now it is known that hypertension is a common complication of the anti-VEGF-signaling pathway therapy, which is the best effective treatment strategy

of the malignancy. Therefore, the incidence and severity of hypertension are dependent mainly on the type and the dose of the anti-VEGF drugs. But exact molecular mechanisms that lead to hypertension in subjects treated with anti-VEGF are still unclear, although endothelial dysfunction and increased vascular resistance, due to impaired nitric oxide production, reduced prostacyclin synthesis, endothelin-1 upregulation, oxidative stress, and rarefaction of vessels have been noted. Results of recent studies have been allowed to suggest that the therapeutic use of the VEGF antagonist sunitinib is able to induce hypertension through Rho kinase (ROCK) inhibition in the nephron that leads to increase of renal vascular resistance (RVR) and renal sodium reabsorption (Grisk et al. 2014). Overall, VEGFR may regulate renal sodium absorption and attenuate vasomotion. In this context, anti-VEGF-1-induced hypertension is considered a model of primary sodium retention deterioration associated with increased RVR. Therefore, anti-VEGF drugs may suppress metabolism of podocytes and thereby lead to their dysregulation, proteinuria, and hypertension (Hayman et al. 2012). Thus, blocking of VEGF-signaling pathway is key mechanism of hypertension in patients with advanced or recurrent malignancy that underwent chemotherapy. Unfortunately, there is a lack of evidences regarding effective methods for prediction of hypertension onset in anti-VEGF drugs-treated patients based on serial measurements of VEGF-1 or VEGFR in blood.

Systemic Hypertension and Circulating VEGF-1

There are attempts to use circulating VEGF-1 level as biomarker of clinical evolution of hypertension in small patient cohorts. It is known that VEGF level in the pregnant woman is significantly higher than in the normal female population matched by age. Although circulating level of VEGF-1 in hypertensive patients is low, there are evidences that increased VEGF-1 levels in pregnant women with severe hypertension may be discussed a risk of preeclampsia and predictor of impaired fetal growth, as well as VEGF level in the serum is negatively related to disease condition (Zawiejska et al. 2014; Wender-Ozegowska et al. 2014). Based on the idea that placental oxidative stress may be a key intermediate step in the pathogenesis of (pre)eclampsia and that it has been associated with excessive secretion of various antiangiogenic factors, VEGF-1 is considered a candidate for predictive biomarker among pregnancy-related hypertension. Lacchini et al. (2014) reported that VEGF-1 polymorphisms is associated well with cardiac remodeling and left ventricular hypertrophy in hypertensive patients. Moreover, genotypes for VEGF-1 polymorphisms can be useful to help identify hypertensive patients at greater intrinsic risk for heart failure. Some pregnancy-related antihypertensive drugs (methyldopa) may affect placental vascularization and prevent gestosis by increased VEGF concentration (Xu et al. 2014). Probably we need novel investigations to be understanding of the predictive role of the VEGF-1 in prehypertension and hypertensive state.

VEGF-1 and Pulmonary Arterial Hypertension

Pulmonary arterial hypertension is considered a result in dysfunctional angiogenesis that lead to obliteration of the lung vessel. The role of proangiogenic factors, such as VEGF-1 and its receptors, remains incompletely understood (Voelkel and Gomez-Arroyo 2015). It has been suggested that hypoxia induce overexpression of VEGF-1 in the endothelial cell of pulmonary arteries and thereby promote proliferative changes of endothelium that directly lead to obliteration of pulmonary vessels and increase of pulmonary pressure. Recent clinical studies have shown that concentration of VEGF-1 in subjects with pulmonary arterial hypertension is mild-to-moderate higher when compared with healthy volunteers. Surprisingly, there is no clinical interrelationship between severity of disease and circulating level of VEGF-1, while for natriuretic peptides, in particular, such association was found (Giannakoulas et al. 2014). In summary, the predictive role of elevated circulating VEGF-1 in patients with pulmonary arterial hypertension is still not clear.

VEGF-1 and Cardiac Dysfunction in Myocarditis

Mechanisms of cardiac dysfunction in myocarditis have not been fully elucidated. Though it remains controversial whether angiogenesis is beneficial or harmful in inflammatory disease, significant vascular destruction might possibly impair cardiac function in myocarditis (Tada et al. 2014; Huusko et al. 2010). It has been suggested that neovascularization supported by overexpression of VEGF-1 could improve cardiac function in myocarditis through suppression of oxidative stress (Jain et al. 2013). These data indicated that overexpression of VEGF-1 appears not only as the ability to regulate cardiac remodeling, as well as contributes to prevent the development of postmyocarditis dilated cardiomyopathy (Arumugam et al. 2013). Although recent studies have shown that the increased level of VEGF-1 mRNA has been detected after transient inflammatory and ischemic injury (Banai et al. 1994; Hojo et al. 2000), the predictive role of circulating VEGF-1 mRNA in myocarditis and dilated cardiomyopathies is still not clear.

Predictive Role of VEGF-1 in Acute Stroke

Inflammation is considered as the key mechanism in the pathogenesis of an ischemic stroke and other forms of ischemic brain injury (Zeng et al. 2013; Jin et al. 2013). Irrespective of a number of recent clinical studies, which demonstrated an indirect interrelation between circulating proinflammatory cytokines and a cardiovascular risk in hypertensive patients after an ischemic stroke, the effect of low intensity proinflammatory activation on modulation of recurrent cardiovascular events is still understood and controversial (Arenillas et al. 2003; Luo et al. 2012; Tuttolomondo et al. 2012). Proinflammatory cytokines were postulated to be able to modulate the

activity of endothelial cells via induction of synthesis of VEGF (Ferrara et al. 2003; Orecchia et al. 2003).

It has been known that VEGF-1 improved blood-brain barrier integrity (Shen et al. 2011). While an induction of VEGF-1 on endogenous neurogenesis and angiogenesis is known, the innate mechanisms of atherothrombotic-related evolution of brain injury and activated endogenous repair mechanisms are not fully understood. The production of VEGF-1 due to focal brain ischemia was found to be able to create a neuroprotection, to improve neoangiogenesis and neurogenesis (Sun et al. 2003; Hayashi et al. 1998). Therefore, VEGF-1 is able to induce postischemic neurovascular remodeling and apoptosis (Hermann and Zechariah 2009; Kim et al. 2013). Probably, these mechanisms underlie the derangement of progressive three-dimensional perivascular cytoarchitectonics, expanding the penumbra zone and worsening cerebral ischemia (Lo 2008). Since the angiopoetic VEGF-1 effect is systemic, it might be assumed that neovascularization in the vulnerable atheroma site should promote progressive worsening of the mechanical capacity of the atheroma cap, the formation of the phenomenon of "fatigue" cap, the appearance of endothelial dysfunction, and deregulation of vascular tone, which ultimately leads to a corresponding atherothrombotic events in any vascular territories (Testa et al. 2008). It has been supposed that immediate VEGF-1 effects are probably adaptive in nature in hypertensive patients after ischemic stroke, while delayed VEGF-1 effects may be associated with recurrent clinical events, in particular, mediated by atherothrombosis (Berezin and Lisovaya 2014a, b).

In this context, clinical studies are required, probably, using comparison various biological markers, including VEGF-1. Recent investigations have really revealed that some biological markers of endothelial dysfunction, such as VEGF-1, and some indicators of proinflammatory activation have a predictive value for clinical outcomes in patients at high cardiovascular risk only (Khurana et al. 2013; Adams et al. 1993; Ridker et al. 2001, 2008). It has been hypothesized that the predictive value of the repeatedly measured circulating VEGF-1 level will be better than single peak VEGF-1 level for predicting of recurrent cardiovascular events in hypertensive patients after an ischemic stroke (Berezin and Lisovaya 2014a). The preliminary results of the small studies appear to be optimistic for use of VEGF-1 monitoring in acute stroke patient with further risk stratification (Berezin and Lisovaya 2014a, b). Based on ROC-analysis, authors observed the optimal cutoff points of circulating VEGF-1 level in acute ischemic stroke patients at baseline (Model 1) and at 6-month follow-up (Model 2) were found to be 403.57 pg/ml and 450.15 pg/ml, respectively. For these cutoff points, sensitivity and specificity were 78.6 % and 70.0 %, as well as 85.7 % and 70.5 % in terms of the positive and negative likelihood ratio equal to 1.12 and 0.305, as well as 2.86 and 0.202, respectively. At the same time, areas under ROC curve (AUC) for both models were 0.76 (95 % CI = 0.602–0.917; $P = 0.001$) and 0.824 (95 % CI = 0.707–0.921; $P = 0.001$). This result showed a higher predictive value of Model 2 compared with Model 1 (Fig. 1). Authors reported that increased VEGF-1 concentrations to be not only associated with a higher incidence of recurrent cardiovascular events (Fig. 2) but it may be a reflection of the phenomenon of progressive vascular remodeling in a long-term period.

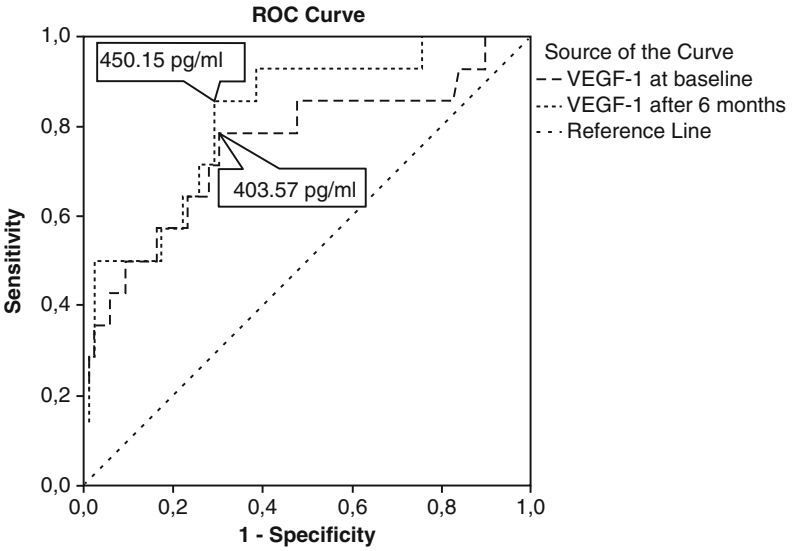

Fig. 1 Cutoff points of the VEGF-1 concentration at baseline and after a 6-month follow-up with the optimal predictive value for cumulative cardiovascular events (Adopted from Berezin and Lisovaya 2014b)

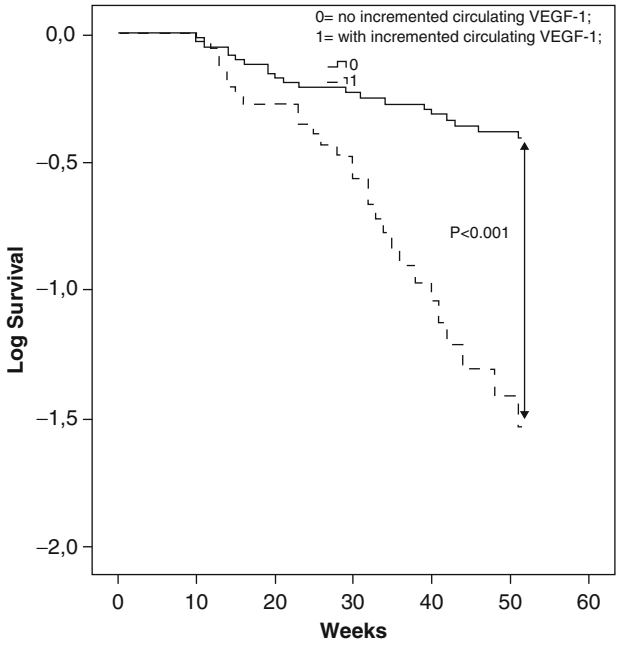

Fig. 2 The results of the Kaplan-Meier analysis with respect to the occurrence of cumulative clinical events depending incremented circulating VEGF-1-within 6 months of observation period (Adopted from Berezin and Lisovaya 2014b)

Thus, VEGF-1 level in acute ischemic stroke patients might be have a value for at risk stratification. On the other hand, data regarding VEGF-1 in patients with other types of stroke, including intracranial hemorrhage, is very limited.

Potential Applications to Prognosis, Other Diseases, or Conditions

Although there are no sufficient evidences that the clinical correlations of circulating levels of VEGF-1 in subjects with cardiovascular diseases might have predictive value, it has been suggested that exaggerated VEGF-1 level would confer a better prognosis in CAD patients, especially those who underwent revascularization procedures or have acute/acutely decompensated heart failure due to ischemic and inflammatory reasons. By now, data for potentially negative effect of VEGF-1–related neovascularization in plaque region might be considered a mechanism of vulnerability of patient (Fig. 3). Therefore, the negative effect of VEGF-1 on progression in age-related diseases, such as early diabetic retinopathy, has been reported. Currently the continued monitoring for changes in VEGF-1 level is not recommended, but vulnerable patient populations at high cardiovascular risk, probably, may have some benefit in prediction of clinical outcomes based on serial assessment of circulating VEGF-1 (Table 3).

Summary Points

- VEGF-1 is a heterodimer with a glycoprotein structure that belongs to the superfamily of vascular endothelial growth factors synthesized by a wide spectrum of circulating cells and possessed a key player in the processing of

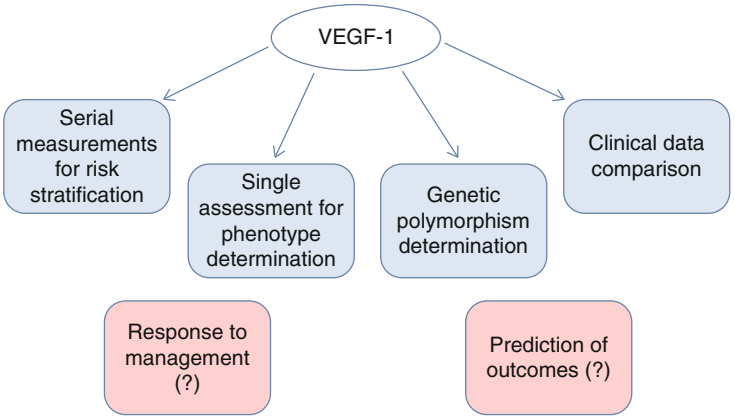

Fig. 3 VEGF-1 as biomarker of cardiovascular vulnerability of the patients. Figure shows that VEGF-1 may be useful as biomarker of cardiovascular vulnerability of the patients. Although there is no evidences on the predictive role of VEGF-1 determined in large randomized clinical trials, the perspectives of VEGF-1 use in risk stratification appears to be optimistic

revascularization neoangiogenesis, reperfusion, as well as neuroprotection and cardioprotection.

- Hypoxia and inflammation are considered the main inductors overexpression of VEGF-1 that realize their proliferative effect through involvement of glycoprotein 130- glycoprotein 130 ligand system and nuclear transcription factors.
- The main source of the synthesis of VEGF-1 in cardiovascular disease is probably not ischemic tissue, but circulating platelets and mononuclears are.
- VEGF-1 is able to induce postischemic neurovascular remodeling and apoptosis that lead to derangement of progressive three-dimensional perivascular cytoarchitectonics expanding the penumbra zone and worsening cerebral ischemia.
- In patients with acute myocarditis and dilated cardiomyopathy neovascularization, supporting by overexpression of VEGF-1 could improve cardiac function in myocarditis through suppression of oxidative stress.
- The clinical correlations of circulating levels of VEGF-1 in subjects with cardiovascular diseases are largely unclear
- The circulating level of VEGF-1 in the majority of clinically stable patients with cardiovascular diseases is under analytical detection limits, that is, an internal limitation for monitoring of this biomarker.
- Among unstable subjects with acute coronary artery disease (CAD), especially required interventional procedures, acute myocarditis and dilated cardiomyopathy with acute or acutely decompensated heart failure, circulating VEGF-1 might be detected in peripheral bloodstream in exaggerated level.
- The monitoring of VEGF-1 in separately vulnerable patient populations at high cardiovascular risk, probably, may have a significant value for prediction of clinical outcomes, but this assumption is required evidence obtained in large clinical trials.

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Macrophage Metalloprotease (MMP)-12 as a Cardiovascular Biomarker

16

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_29

Abstract

Cardiovascular diseases (CVD) worldwide represent the principal cause of death. Thus, the challenge to identify novel and clinically useful biomarkers of CVD risk has focused the attention over the last years. Atherosclerosis (ATS) is one of the major causes of CVD. ATS is an inflammatory multifactorial disease, in which the complex interaction between immune cells and inflammatory mediators drives the growth of atherosclerotic lesions and their progression toward complications. Taking into account the great number of molecules and cells involved, over the time several markers have been evaluated, including inflammatory mediators, acute phase response proteins, blood cells and proteins implicated in lipid metabolism. The study of these molecules has significantly contributed to improve the knowledge about the immune-inflammatory mechanisms involved in the pathogenesis of ATS; however, they did not often represent useful biomarkers in the clinical practice due to their poor specificity. The contribution of matrix metalloproteinases (MMPs) to CVD has been extensively reported, whereas their role as biomarkers and prognostic factors is not fully elucidated. Here we point out the role of MMP-12 as biomarker of CVD.

Keywords

Cardiovascular diseases • Atherosclerosis • Metalloproteases • MMP-12 • Biomarkers • Athero-occlusive diseases • Aortic aneurysm and dissection

Abbreviations

AAA	Abdominal aortic aneurysm
AAD	Acute aortic dissection
ACS	Acute coronary syndromes
AMI	Acute myocardial infarction
AOD	Vascular occlusion
ATS	Atherosclerosis
CAD	Coronary artery disease
CAS	Carotid artery stenosis
CKD	Chronic kidney disease
CRP	C-reactive protein
CVD	Cardiovascular diseases
ECM	Extracellular matrix
MMPs	Matrix metalloproteinases
SAP	Stable angina pectoris

SD	Sudden death
SMCs	Smooth muscle cells
TEA	Thromboendarterectomy
TIA	Transient ischemic attack
UAP	Unstable angina pectoris
VEGF	Vascular endothelial growth factor
VSMCs	Vascular smooth muscle cells

Key Facts of Cytokines

- Cytokines represent humoral mediators of immune system released during immune response.
- Cytokines are secreted by several cell types, including endothelial, immune, epithelial, and mesenchymal cells.
- Each immune cell type releases a characteristic set of cytokines which often allow us to recognize the main cellular population involved in each inflammatory process. However, an overlapping release of the same cytokine by different cell type occurs.
- Cytokines are deeply involved in inflammatory process underlying several pathological conditions, ranging from wound healing to cancer.
- Cytokines represent an interesting target in the clinical practice, so that their inhibitors are effective in the treatment of inflammatory disease such as rheumatoid arthritis.
- Cytokines represent key mediators of atherosclerotic disease from its early stage to its complications.

Definitions

Animal models Mice or rabbit wild type or genetically modified used to perform experimental study in vivo.

Athero-occlusive diseases Atherosclerosis of artery wall characterized by lumen occlusion.

Biomarker Molecule or substance involved in a pathological process, which levels can identify patients affected by such specific disease.

Dilatative disease Aortic wall dilatation.

Multifactorial disease A disease in which genetic predisposition and environmental factors collaborate to determine clinical manifestations.

Introduction

Cardiovascular diseases (CVD), which also include cerebrovascular diseases, worldwide represent the principal cause of death (van Holten et al. 2013; Table 1). Thus, the challenge to identify novel and clinically useful biomarkers of CVD risk has focused the attention over the last years.

Atherosclerosis (ATS) is one of the major causes of CVD. ATS is an inflammatory multifactorial disease, in which the complex interaction between immune cells and inflammatory mediators drives the growth of atherosclerotic lesions and their progression toward complications (Hansson 2005). Adhesion molecules, pro-inflammatory cytokines, chemokines, hydrolytic enzymes, and growth factors are all involved in this process (Charo and Ransohoff 2006). Endothelium injury and inflammation are considered key events in the early phases of ATS process. Endothelial activation, indeed, leads to altered vessel permeability, increased leukocyte adhesion, and chemokine, cytokine, and metalloproteinase (MMP) release, triggering and maintaining an inflammatory state, which is responsible for parietal remodeling (Charo and Ransohoff 2006). Regarding this process, it has been demonstrated that immune response strongly influences the outcome of intraparietal inflammation toward wall occlusion or dilatation (Aukrust et al. 2008; Xu et al. 2001). Despite sharing the same risk factors, athero-occlusion (AOD) and acute aortic dissection (AAD) diverge greatly in their immunological pattern: lymphocyte T $CD4^+$ (T helper 1) activation has been mainly associated with proliferative features of plaque development (Hansson 2005; Charo and Ransohoff 2006), whereas macrophages have been related to the lytic environment involved in AAD (Del Porto et al. 2010; Butcherand and Galkina 2012).

Athero-occlusive Diseases

Atherosclerotic lesions include a wide spectrum of wall alterations which range from the lipidic stria to complicated plaque. Histological features of ATS start with a lipid core which is progressively enriched with immune cells. Initially macrophages

Table 1 Epidemiology of CVD. Overall epidemiology of CVD in Europe (Data from European Heart Network and European Society of Cardiology, September 2012)

CVD	Incidence	Mortality
Coronary heart disease	154/100,000/year	489,000/year
Cerebrovascular disease (stroke)	76/100,000/year	208,000/year
Congestive heart failure	670,000/year	277,000/year
Myocarditis	5/100,000/year	1/100,000/year
Endocarditis	12/100,000/year	2/100,000/year
Cardiomyopathy	8/100,000/year	4/100,000/year
Abdominal aortic aneurysm	13/100,000/year	15,000/year
Acute aortic dissection	3/100,000/year	0.6/100,000/year

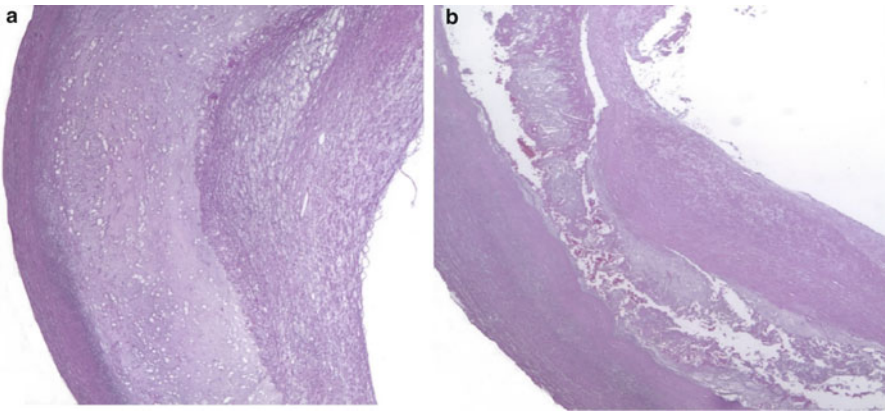

Fig. 1 Atherosclerotic plaques. Histological sections of uncomplicated plaque (panel **a**) and complicated plaque (panel **b**). Hematoxylin-eosin staining, 2.5× magnification

represent the main cellular population detectable inside the plaque; subsequently, the activation of the immune response causes T helper and B lymphocyte recruitment, which progressively achieves antigen-specific activity (Hansson 2005). Neoangiogenesis participates in the maintenance of chronic inflammation by promoting further leukocyte recruitment and by amplifying pro-inflammatory Cytokine and MMP release. Neoangiogenesis, therefore, represents a thorn for the plaque instability (De Boer et al. 1999). Fibrous cap formation is the last step of plaque growth and protects it from disruption (Jones et al. 2003). The evolution of the atherosclerotic plaque from the fatty streak to advanced plaque is also associated with an increase in its content of collagen (Stary et al. 1995), in the number of smooth muscle cells (SMCs) (Brown et al. 1997), and in levels of MMP-2 and -9 (Jones et al. 2003). Plaque complications include rupture or ulceration, with consequent thrombotic apposition, fibrous cap fissuring, and hemorrhage within the atheroma (Stary et al. 1995). Plaque instability, which also depends on immune response and inflammation, underlies such complications which in turn are responsible of clinical manifestations (Fig. 1). The immunological process underlying plaque development, growth, and complications is extremely complex and needs the contribution of different branches of the immune response. Such immune cross-talk is controlled by regulatory T cells, which act on several effectors, including macrophages, resident cells, and B and T lymphocytes. As mentioned above, atherosclerotic plaque development is related to $CD4^+$ T helper response (Hansson 2005). $CD4^+$ cells include T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17), regulatory T helper (T regs), and CD28- subpopulations, which differ in their functions and products. An imbalance between Th1 and Th2 subpopulations toward a Th1 response has been reported in CVD patients (Hansson 2005). Moreover, the expansion of pro-inflammatory T helper subsets, such as Th17 and $CD4^+CD28^-$, has been described in patients both with symptomatic coronary artery and carotid artery disease (Liu et al. 2012). Accordingly, an imbalance of the ratio Th17/Tregs has been related to the extent of ATS disease (Figs. 2 and 3).

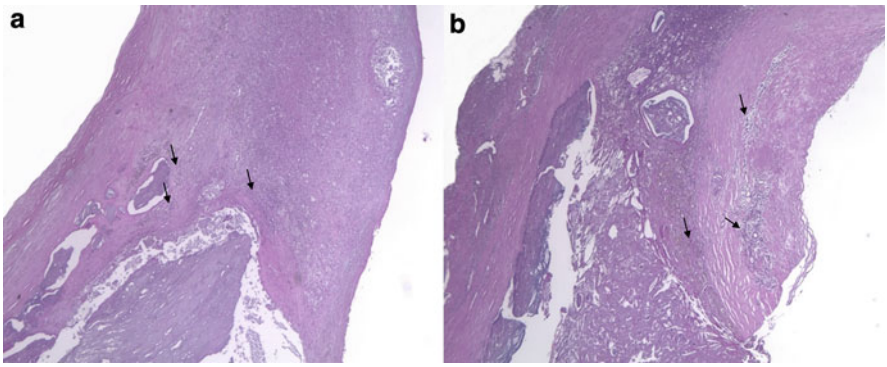

Fig. 2 Inflammatory lymphomononucleate infiltration in atherosclerotic plaques. Histological sections of uncomplicated plaque (panel **a**) and complicated plaque (panel **b**). Hematoxylin-eosin staining, 2.5× magnification. The *arrows* indicate the inflammatory lymphomononucleate infiltration

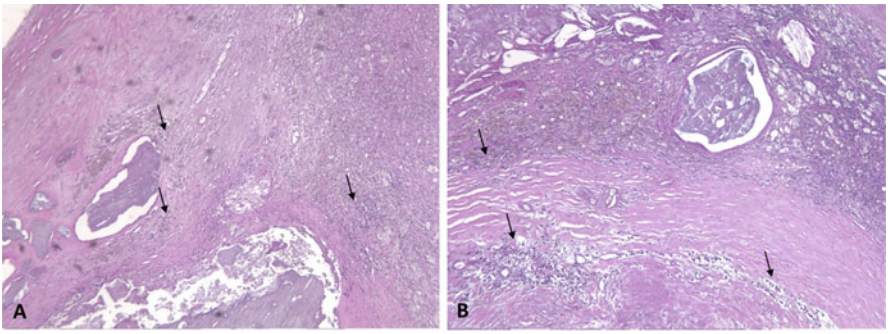

Fig. 3 Inflammatory lymphomononucleate infiltration in atherosclerotic plaques. Histological sections of uncomplicated plaque (panel **a**) and complicated plaque (panel **b**). Hematoxylin-eosin staining, 5× magnification. The *arrows* indicate the inflammatory lymphomononucleate infiltration

Several endogenous and exogenous triggers, including traditional cardiovascular risk factors and infectious agents, can interfere with plaque evolution by promoting the establishment of a pro-atherogenic immune pattern. Plaque history, therefore, depends on several factors such as nature of the trigger, preexisting pro-inflammatory state and pattern of immune response at time of exposure, which all contribute to plaque formation, growth, and complications.

Aortic Aneurysm and Dissection

Aortic wall diseases include aneurysm and dissection and may involve all segments of aorta: ascending aorta, arch, or descending thoracic aorta. AAD is a life-threatening disease with an incidence of about 2.6–3.6 cases/100,000/year

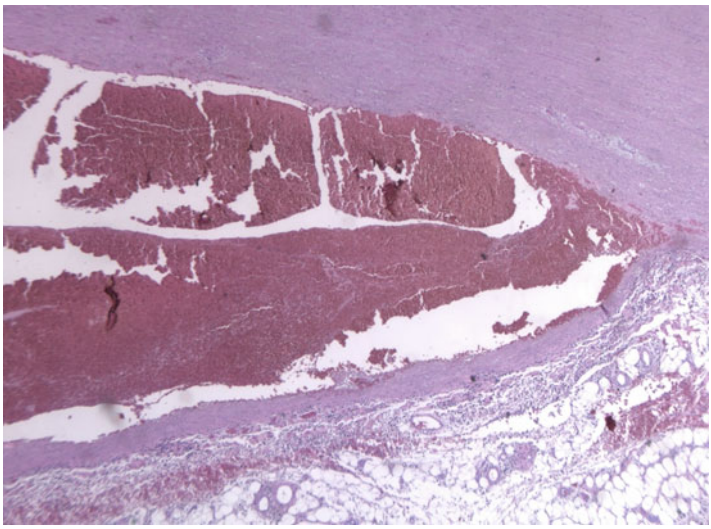

Fig. 4 Aortic dissection. Histological section of aortic dissection. Hematoxylin-eosin staining, 2.5× magnification

(Isselbacher 2005). It is characterized by medial degeneration with intima tear and crossing of blood into the artery wall, which causes the formation of a false lumen within the tunica media (Fig. 4). Depending on the site of rupture, AAD is classified as Stanford-A type when the ascending aortic thoracic tract and/or the arch is involved and Stanford-B type when the descending thoracic aorta and/or aortic abdominal tract is targeted. Stanford-A AAD is the most frequent and occurs in almost 75 % of total cases with a mortality reaching 90 % if untreated. Medial degeneration, which is the main histological finding associated with aortic aneurysm and dissection, consists of SMCs depletion, elastic fiber fragmentation, and collagen degradation, which results in ECM weakening (Didangelos et al. 2011). Both genetic and acquired conditions have been associated with medial degeneration, including inherited connective tissue diseases, ATS, and arterial hypertension (Isselbacher 2005). It was generally believed that genetic and inflammatory factors contribute differently to medial degeneration in the different parts of the aortic wall. Inherited connective tissue diseases have been mainly related to Stanford-A aneurysm and dissection, whereas ATS has been mostly related to Stanford-B (Isselbacher 2005).

Recently, it has been demonstrated that inflammation underlies to wall weakening in each aortic tract, including the ascending (Del Porto et al. 2010). However, it has been observed that aortic dilatation and rupture arise through immune-inflammatory mechanisms that differ, at least in part, from those responsible for athero-occlusive disease. Aortic aneurysms, indeed, have been mostly associated with a Th2 activation (Aukrust et al. 2008), whereas aortic rupture has been related to macrophages activation and matrix-degrading protein release (Proietta et al. 2014). A further confirm of the pivotal role of macrophages in aortic rupture has been pointed out

from experimental models. It has been demonstrated that in apoE-/E- mice undergoing to continuous angiotensin I infusion dissection occurred first, within the first 5 days, and was characterized by intraparietal macrophage recruitment. Survivor mice developed aneurysms, whereas plaque formation occurred last and was accompanied by a progressive increase in the number of T lymphocytes that infiltrated the arterial wall (Saraff et al. 2003). Interestingly, also a distinguishing cytokine pattern has been associated with aortic wall diseases. A hyperexpression interleukin (IL)-6 and IL-8 has been described both in patients with Stanford-A AAD and in those with abdominal aortic aneurysm/dissection (AAAs), strongly suggesting that these two cytokines drive intraparietal inflammation toward dilatation or rupture in the early phases of the atherosclerotic diseases (Lindeman et al. 2008). Macrophage products have been demonstrated to play a central role in weakening aortic wall, so that IL-6, IL-8, and MMP-12 have been recognized as distinguishing markers of aortic disease (Curci et al. 1998; Longo et al. 2005; Ikonomidis et al. 2006).

Matrix Metalloproteases (MMPs)

MMPs include a wide spectrum of zinc-dependent proteolytic enzymes, which are involved in the breakdown of ECM, being able to degrade collagen and elastin fibers (Chen et al. 2013). ECM is the combination of extracellular macromolecules which encloses and contains resident cells and plays a key role for the proper functions of the different organs of the human body, including the heart and vessels. Normal arterial wall remodeling is characterized by a balance between the levels of MMPs and their tissue inhibitors (TIMPs) that provides the appropriate rates of destruction/synthesis of ECM necessary to maintain health of the arterial wall (Chen et al. 2013). An imbalance between the production MMPs and TIMPs has been demonstrated to play a key role in the pathogenesis of several diseases including AOD (Charo and Ransohoff 2006), aneurysms (Didangelos et al. 2011), post-angioplasty restenosis, and heart failure (Spinale and Villarreal 2014).

Several factors are able to induce MMPs release. It has been demonstrated that MMP-9 values principally increase subsequently to shear stress, whereas MMP-2 levels mainly increase after oxidative stimuli. Hypoxia induces both MMP-2 and MMP-9 raise, whereas pro-inflammatory cytokines such as IL-17 induce tumor necrosis factor (TNF)-α and IL-18 favorite MMP-9 discharge (Chen et al. 2013). Thus, it is well understandable that MMP-2 and MMP-9 are deeply implicated in promoting ATS progression and complications.

It has been demonstrated that MMPs, due to their own activities, play an opposite role on plaque development. They, indeed, are able to promote plaque formation by favoring the migration of vascular SMCs (VSMCs) into the intimal space, by inducing neoangiogenesis (Stary et al. 1995) and by facilitating leukocyte recruitment, but also MMP activity may diminish plaque volume by degrading intimal ECM and by digesting the external elastic lamina, which minimize luminal encroachment (Jones et al. 2003). Accordingly, MMP activities promote also aortic

wall rupture. Thus, the contribution of MMPs and TIMPs to ATS progression seems to be not limited to the sole degradation functions. MMPs, indeed, are able to interact with cytokines, chemokines, and cell surface proteins and to regulate trafficking, migration, proliferation of cells, and apoptosis (Sternlich and Werb 2001).

Experimental models have also confirmed that MMP-2 and MMP-9 release related to angiotensin II (AG II) infusion is involved both in aneurysm and plaque formation. Interestingly, AGII has been demonstrated to be able to activate MMP-8 and MMP-13 into atherosclerotic lesions, inducing plaque destabilization (Cheng et al. 2009). All these observations strongly suggest that different MMPs are involved in the different steps of ATS lesion evolution. Further studies are needed to clarify which and when each MMP is activated during the ATS process and what are their relationship with ATS evolution.

Considering the prevalence of ATS and its complications and their relevance in the clinical practice, the identification of early markers of CVD has represented a challange. Taking into account the great number of molecules and cells involved, over the time several markers have been evaluated, including inflammatory mediators, such as cytokines and chemokines; acute phase response proteins, including C-reactive protein (CRP), pentraxin, and fibrinogen; blood cells; and proteins implicated in lipid metabolism (Aiello and Kaplan 2009; van Holten et al. 2013). The study of these molecules has significantly contributed to improve the knowledge about the immune-inflammatory mechanisms involved in the pathogenesis of ATS; however, they often did not represent useful biomarkers in the clinical practice due to their poor specificity. Pro-inflammatory cytokines and CRP levels, indeed, are increased in ATS, but also they raise not specifically in several chronic inflammatory disease such as autoimmune syndromes, tumors, and infections. Moreover, elevated values of acute phase reactants are detectable both in coronary syndromes and stroke and AAD (Aiello and Kaplan 2009; Casas et al. 2008). Analogously MMP-2 and MMP-9 levels raise in arterial hypertension, in ischemic cardiac disease, in carotid artery stenosis, and in Stanford-A and B aortic aneurisms/dissection, being related to ATS risk in the whole, more than to its different clinical features (Sangiorgi et al. 2006). Sometimes, instead, sensitive and specific markers of CVD have been recognized such as troponin I, which represents the more successful indicator of acute coronary syndromes (ACS). Recently a specific role has been also recognized for MMP-12 in aortic aneurysms and dissection.

MMPs: Structure and Functions

MMPs belong to a subfamily within the superfamily of metalloendopeptidases $Zn2^{+}$-dependent metzincins (Chen et al. 2013). These enzymes are produced and secreted by several immune and resident mesenchymal cells constituting the vascular wall, including T lymphocytes, macrophages, endothelial cells, and SMCs. More than 20 MMPs have been identified and further subdivided into collagenases, gelatinases, elastases, and stromelysins based on their structure and specific

substrates, which often happen to overlap. All MMPs exhibit the same modular structure that includes from N- to C-terminus: the signal sequence (or pre-domain), zymogenic pro-peptide (pro-domain), an active catalytic domain (with a zinc-binding region), and hemopexin-like domain (Chen et al. 2013). The pre-domain, indispensible for secretion, is removed after directing protein synthesis to the endoplasmic reticulum; the ∼80-residue pro-domain maintains enzyme latency until it is removed or disrupted; the ∼165-residue zinc- and calcium-dependent catalytic domain dictates cleavage-site specificity (Chen et al. 2013); and the hemopexin-like domain works for collagen binding, pro-MMP activation, and dimerization. Although all MMPs share this modular combination, specific domains are inserted in some MMPs.

MMPs are transcriptionally regulated: various cytokines, such as IL-1 and TNF-α, induce MMP transcription through the activation of different intracellular signaling cascades. In addition to transcription-level regulation, most MMPs are translated as zymogen inactive forms (Chen et al. 2013). The proenzymes are secreted and subsequently activated, either by another protease or by autoactivation, in the extracellular space where MMPs principally perform their biological functions. Inhibitors of MMPs represent a further level of regulation for the activity of these enzymes. TIMPs are the most specific endogenous MMP inhibitors and show a considerable overlap in their ability to target different MMPs, although there are some differences in the affinity of specific inhibitor-protease pairs (Khokha et al. 2013). In particular, TIMP-1 inhibits MMP-1, MMP-3, MMP-7, and MMP-9. TIMP-2 inhibits MMP-2, whereas TIMP-3 is reported to decrease activities of MMP-2 and MMP-9. TIMP-4 on the other hand inhibits MT-MMP and MMP-2 activity. MMPs inhibition in vivo also occurs through relatively nonspecific inhibitors such as α-2 macroglobulin (Chen et al. 2013). Moreover, exogenous inhibitors to MMPs, such as the tetracyclines, are artificial MMP inhibitors that can blunt their activity.

MMPs perform numerous biological functions through their ability to degrade matrix components. In particular MMPs are involved in tissue remodeling, neoangiogenesis, cell mobility and migration, and release of cytokines, growth factors, and chemokines. Thus, when aberrantly or excessively expressed, MMPs cause such tissue destruction, which occurs in the different pathological conditions, such as emphysema, arthritis, cancer, inflammation, neurodegenerative diseases, liver diseases, chronic kidney disease (CKD), and CVD (Hua et al. 2009).

MMPs and Cardiovascular Diseases

Coronary Heart Diseases

Acute coronary syndromes (ACS) are associated with high rates of mortality and disability and represent one of the main causes of severe CVD. ACS is classified as either acute myocardial infarction (AMI) or unstable angina pectoris (UAP),

depending on whether vulnerable plaque determines complete or incomplete artery occlusion. Stable angina pectoris (SAP) and at least a part of sudden deaths (SD) also depend on coronary artery ATS. Instability of atherosclerotic plaques plays an important role in the pathogenesis of both cerebrovascular disease and coronary heart disease (Muller et al. 2014). The formation of a fibrous cap is an important step in atherogenesis. Cap, indeed, stabilizes the lesion and prevents plaque rupture, thus avoiding thrombus formation and occurrence of adverse clinical events such as TIA/stroke and UAP/AMI; however, enhanced fibrogenesis contributes also in narrowing the lumen, which potentially results in chronic ischemia. The strength of the fibrous cap depends on a dynamic balance between collagen synthesis and degradation. In this context, MMPs can enhance plaque vulnerability, potentially turning plaque from a stable to an unstable phenotype, so that an association between MMPs/TIMPs activity and plaque rupture has been reported (Guo et al. 2014). Actually, it has been demonstrated a localization of MMPs in the shoulder region of vulnerable lesions (Jones et al. 2003). Several MMPs, such as MMP-1, MMP-2, and MMP-9 (Hojo et al. 2001) and their specific TIMP (TIMP-1), have been associated with ACS. In particular, a twofold increase of MMP-2 levels has been described in UAS and AMI patients versus SAP and healthy subjects (HS) (Hojo et al. 2001). Moreover, an increase of intracellular MMP-9 levels has been observed in AMI (Brown et al. 1997). To further confirm the pivotal role of MMPs in the pathogenesis of ACS, it has been observed that patients with AMI had highest serum levels of MMP-9 that progressively decreased in UAP and SAP patients (Guo et al. 2014). Finally, a role has been also recognized for MMP-2, MMP-7, and MMP-9 in myocardial remodeling and post-ischemic heart failure.

A role for TIMP-1 and TIMP-2 has also been recognized, suggesting that not only MMPs increase but the ratio MMPs/TIMPs is deeply involved in vascular remodeling underlying plaque vulnerability.

Carotid Artery Stenosis (CAS)

Several experimental evidences have confirmed that MMPs are deeply involved also in carotid atherosclerotic plaque development, growth, and complications, suggesting that a deregulation of MMPs/TIMPs functions occurs also in cerebrovascular diseases (Kunte et al. 2010). Among all MMPs, a pivotal role in promoting carotid plaque remodeling has been proposed for MMP-2 and MMP-9 both in humans and in mice. In particular, it has been demonstrated that MMP-9 levels increase in the early phases of plaque development and are related to VSMC proliferation and their migration inside intima (Johnson et al. 2011). Also MMP-3 has been demonstrated to increase carotid plaque size by promoting VSMC recruitment. However, this action on VSMCs produces a double action on carotid artery lesion: increases intimal thickness and contemporary stabilizes plaque. In agreement, it has been observed that inhibition of MMP-3 and MMP-9 functions underlies both

to reduced plaque growth and to increased plaque vulnerability (Johnson et al. 2011). MMP-1 has also been involved in wall remodeling of CAS both symptomatic and asymptomatic. A different distribution of MMPs has been observed inside atherosclerotic plaque at any site of the arterial tree including carotid and coronary arteries. MMP-1, MMP-3, MMP-7, MMP-9, and MMP-12 are expressed by macrophage in the shoulder region of the plaque and the border between lipid core and fibrous areas, suggesting for them a role in plaque remodeling. Actually, an increase in gene expression of MMP-1, MMP-9, MMP-12, and MMP-14 has been observed in vulnerable plaque and has been associated with plaque instability (Muller et al. 2014). Finally, MMP-2, MMP-3, and MMP-9 have also been related to restenosis risk after thromboendarterectomy (TEA) (Busti et al. 2010).

Aortic Aneurysms

Abdominal aortic aneurysm is an inflammatory-degenerative disease, characterized by aortic wall weakening and dilatation (Zhang et al. 2014). Aneurysm formation can occur at any aortic site and derives from a complex process involving vascular, immune, and mesenchymal cells. Specific changes in the aortic wall, including chronic adventitial and medial inflammatory cell infiltration, decrease in elastin content, and loss of integrity of ECM, have been described by histology (Maegdefessel et al. 2014). Moreover, an inflammatory infiltrate constituted by macrophages, T and B lymphocytes, has been observed within the tunica media in aneurysms. The complex interaction among T lymphocytes, macrophages, and mesenchymal cells induces cytokines, chemokines, and MMP release, which deeply affect parietal integrity. Several MMPs have been involved in the process underlying aneurysm formation; in particular an overexpression of MMP-1, MMP-2, MMP-3, MMP-9, MMP-12, and MMP-13 has been demonstrated both in plasma and within the wall in patients with both thoracic and abdominal aneurysms (Keeling et al. 2005). Accordingly an unbalance between MMPs and TIMPs has been related to aortic wall diseases (Koullias et al. 2004; Flondell-Site et al. 2010; Didangelos et al. 2011). MMP-2 and MMP-9 have been the most MMPs studied in patients with aneurysm occurring at any aortic site, and it has been demonstrated that their levels were correlated to wall weakening (Maegdefessel et al. 2014). However, recently it has been focused the attention on the role of MMP-12 as specific marker of aortic diseases. In particular, it has been demonstrated both in mice and humans that MMP-12 activities were related to abdominal aneurysms formation and growth (Curci et al. 1998; Matsumoto et al. 1998; Longo et al. 2005).

Aortic Dissection

Since acute aortic dissection is a rare disease, relatively few studies have focused the attention only on the role of a small number of MMPs in its pathogenesis. Several evidences support the hypothesis of an involvement of MMPs in parietal weakening

underlying AAD independently of the site of dissection. In particular, increased levels of MMP-1, MMP-2, and MMP-9 have been described in both Stanford-A and Stanford-B patients (Sangiorgi et al. 2006; Karapanagiotidis et al. 2009), whereas a prominent expression of MMP-8 has been reported in Stanford-A AAD (Li et al. 2010). Moreover, a decrease of TIMP/MMP-2 ratio has been associated with the acute phases of aortic dissection (Manabe et al. 2004), whereas a decrease of and TIMP/MMP-9 has been related to aortic dissection in patients having chronic thoracic aortic aneurysms (Zhang et al. 2014).

MMP-12

MMP-12 belongs to the subgroup of elastases. Differently from the other MMPs, MMP-12 is exclusively secreted by macrophages, and it is predominantly expressed in mature tissue macrophages, so that it is also known as "macrophage elastase." This enzyme, in addition to elastin, degrades a broad spectrum of substrates, including type IV collagen, fibronectin, laminin, vitronectin, proteoglycans, chondroitin sulfate, myelin basic protein, δ-1-antitrypsin, and plasminogen (Chen et al. 2013). Another important function of MMP-12 is to activate MMP-2 and MMP-3, which lead to subsequent degradation of other extracellular matrix proteins (Matsumoto et al. 1998). MMP-12 is secreted from macrophages as a 54-kDa proenzyme or zymogen consisting of common MMP domains. Upon activation, MMP-12 not only cleaves its pro-domain but also has a unique propensity to cleave its C-terminal hemopexin-like domain, resulting in a 45 kDa domain and in the 22-kDa catalytic domain (Shapiro et al. 2003). MMP-12 activity is in part regulated by coagulation proteases, such as thrombin and plasmin, and its release is induced by pro-inflammatory cytokines, such as TNF-α and IL-1β. Physiological functions for MMP-12 have been related to its ability to degrade ECM components. However, it has been reported that MMP-12 can also modulate cytokine and chemokine networks, promote macrophage tissue recruitment (Dasilva and Yong 2008), display antimicrobial activity, and regulate antiviral defense (Shapiro et al. 2003; Marchant et al. 2014). Several pathological conditions, such as aortic aneurysm formation (Curci et al. 1998), atherosclerosis (ATS) (Matsumoto et al. 1998), emphysema (Hautamaki et al. 1997), and rheumatoid arthritis (Wang et al. 2004), have been related to MMP-12 functions.

MMP-12 and CVD

MMP-12, also known as macrophage elastase, represents an interesting potential new marker of cardiovascular risk. Knowledge about the role of MMP-12 in ATS came first from animal models, in which through biochemical and immunohistochemical methods it has been demonstrated that MMP-12 levels were related to the extent of the plaque in aortas of rabbits fed a cholesterol-containing diet (Matsumoto et al. 1998). Successive studies performed on transgenic rabbits confirmed that

MMP-12 accelerates the development of atherosclerotic lesion and favorites its progression from fatty streak to fibrous plaque (Liang et al. 2006; Yamada et al. 2008). A further clarification of its role in ATS derives from studies on apoE/MMP-12 double knockouts mice, which displayed reduced lesion size and fibrous layers, with increased SMCs and reduced macrophage content, conferring a stable histological pattern to plaque. Furthermore, it has been demonstrated that the administration of a selective MMP-12 inhibitor halts atherosclerosis (ATS) development in mice (Morgan et al. 2004; Luttun et al. 2004; Johnson et al. 2011; Scholtes et al. 2012).

These findings have also been confirmed in humans. MMP-12, indeed, has been demonstrated to be absent in healthy arteries, minimally present in early ATS lesions, and strongly expressed in advanced atherosclerotic plaques (Morgan et al. 2004; Liang et al. 2006; Yamada et al. 2008). In agreement, it has been demonstrated that plasma MMP-12 concentrations were increased in patients with coronary artery ATS, although its levels did not correlate with severity of the disease (Jguirim-Souissi et al. 2007). On the other hand, in carotid artery stenosis (CAS), the presence of a subset of macrophages expressing MMP-12 has been associated with plaque vulnerability and adverse clinical outcome (Scholtes et al. 2012).

Analogously to AOD, it has been observed that MMP-12 expression was related to atherosclerotic aneurysm formation both in mice (Longo et al. 2005) and in humans. Moreover, MMP-12 has been found in vivo in macrophages infiltrating aortic aneurysm (Curci et al. 1998).

More recently, the expression of MMP-12 has also been evaluated in Stanford-A AAD, using biochemical and immunohistochemical methods. Measurements were performed in Stanford-A AAD patients undergoing aortic replacement, CAD patients undergoing coronary artery bypass surgery, and 10 healthy subjects (HS). Results showed that MMP-12 serum levels were higher in AAD patients than in HS, whereas MMP-12 aortic wall concentration was higher in AAD group than in CAD. Thus, an interesting specific role has been suggested and confirmed for MMP-12 in the development of AAD (Song et al. 2013). In addition, it has been demonstrated that macrophages play a pivotal role in the inflammatory process characterizing Stanford-A AAD in patients with no genetic predisposition. First, it has been demonstrated that macrophage cytokine levels were significantly increased in patients with Stanford-A AAD compared to patients matched for age, sex, and cardiovascular risk factors (Del Porto et al. 2010), whereas any significant difference was observed in T lymphocyte cytokines. A significant increase of serum MMP-12 and vascular endothelial growth factor (VEGF) levels was also reported (Del Porto et al. 2014) confirming that macrophage-mediated inflammation, neoangiogenesis, and matrix degradation are fundamental steps of aortic wall rupture. Subsequently, MMP-12 levels have been evaluated in patients with Stanford-A AAD and in patients with stable critical CAS compared to patients matched for age, sex, and traditional cardiovascular risk factors (RF), by ELISA. The results showed a significant increase of MMP-12 levels in AAD versus CAS and RF, but not in CAS versus RF, strongly supporting the hypothesis that MMP-12 represents a specific marker of Stanford-A AAD (Proietta et al. 2014; Del Porto et al. 2014). Moreover a significant increase of IL-6 and IL-8 serum levels

was observed in AAD versus CAS and RF confirming a pathogenetic role for these two cytokines in Stanford-A AAD. However, their suitability as markers was weakened from their low specificity, since IL-6 and IL-8 values increase in several other inflammatory conditions, including infections.

Potential Applications to Prognosis and Other Diseases or Conditions

Pathways involved in CVD start a long before clinical symptoms, thus the importance of identifying early diagnostic and prognostic markers. Nevertheless, it is not simple to find molecules that can be sensible and specific. The most useful markers are those that appear in the early phases of the disease, that have predictive positive value, and that can help distinguish between low and high cardiovascular risk. In the field of ATS, several biomarkers related to plaque formation and to its progression have been described. These biomarkers are involved in several phases of the ATS process, including oxidative stress, endothelial dysfunction, inflammation, and immune activation, and are all detectable in serum and plasma (Aiello and Kaplan 2009). Since ATS is the major cause behind CVD, some risk factors such as cholesterol levels, diabetes, smoke habit, body mass index, arterial blood pressure, and familiar predisposition are all useful in suspect the presence of CVD (van Holten et al. 2013).

On the other hand, there are other biomarkers that are strongly associated with a wide spectrum of diseases, but do not have clinical application. For instance, fibrinogen, which is an excellent marker for inflammation and coagulative disorders, increases both in CVD and stroke, and does not play yet any practical role in predicting these two disease or identifying patients with high risk of complications (van Holten et al. 2013). In agreement, cystatin C, which is an emerging marker of renal dysfunction, cannot be considered a specific marker of CVD, since its levels increase in renal dysfunction, which is, in turn, a well-known cause of CVD (Fassett et al. 2011).

Several pro-inflammatory molecules, including cytokines, chemokines, and acute phase proteins, have been implicated in CVD (Aiello and Kaplan 2009). Among them, IL-6 is a known pro-inflammatory cytokine, which plays a direct role in the initiation of other inflammatory factors' synthesis, such as CRP and fibrinogen. Both IL-6 and CRP have been proven to predict CVD among healthy individuals in population-based studies (Wilson et al. 2004). CRP is synthesized in response to infection and injury as an immunological endpoint in the classical complement pathway. Moreover, CRP appears to be produced in response to IL-6 release after endothelium injury in the early stages of ATS and during all inflammatory process underlying plaque growth and its complications, so that it has been suggested that CRP represents a marker for instability and rupture of preexisting atherosclerotic plaques (Casas et al. 2008). Nevertheless, its specific role in the pathogenesis of CVD is still under debate. Furthermore, it should not be neglected that IL-6 levels raise not specifically during several inflammatory process, such as infections, autoimmune disease, and neoplasm. Thus, its use as specific biomarkers for CVD is difficult.

Table 2 MMPs involved in CVD. Summary of MMPs involved in CVD and related references

MMP	CVD	References
MMP-1	Aortic aneurysms and dissections	Koullias et al. 2004; LeMaire et al. 2005; Karapanagiotidis et al. 2009; Flondell-Site et al. 2010; Didangelos et al. 2011; Zhang et al. 2014
MMP-2	ACS	Hojo et al. 2001; Silence et al. 2002
MMP-2	AAA	Davis et al. 1998
MMP-2	Aortic aneurysms and dissections	Koullias et al. 2004; LeMaire et al. 2005; Wilson et al. 2004; Karapanagiotidis et al. 2009; Flondell-Site et al. 2010; Didangelos et al. 2011; Saracini et al. 2012
MMP-2	ATS	Kuzuya et al. 2006
MMP-2	ACS	Jenkins et al. 1998; Hojo et al. 2002
MMP-3	ATS	Silence et al. 2001; Orbe et al. 2003
MMP-3	Thoracic aortic diseases	Karapanagiotidis et al. 2009
MMP-3	Aortic aneurysms and dissections	Koullias et al. 2004; LeMaire et al. 2005; Karapanagiotidis et al. 2009, Flondell-Site et al. 2010; Didangelos et al. 2011
MMP-8	ATS	Fang et al. 2013
MMP-8	Thoracic aortic dissection	Li et al. 2010
MMP-9	CAD	Noji et al. 2001; Silence et al. 2002; Blankenberg et al. 2003
MMP-9	ACS	Blankenberg et al. 2003; Guo et al. 2014
MMP-9	AAA	Hovsepian et al. 2000; Yamashita et al. 2001
MMP-9	Aortic aneurysms and dissections	Koullias et al. 2004; LeMaire et al. 2005; Karapanagiotidis et al. 2009; Flondell-Site et al. 2010; Didangelos et al. 2011
MMP-9	Cerebrovascular disease	Kunte et al. 2010
MMP-9	ATS	Brown et al. 1997; Orbe et al. 2003; Luttun et al. 2004; Hua et al. 2009; Tretjakovs et al. 2012
MMP-9	Aortic dissection	Schneiderman et al. 1998; Koullias et al. 2004; Manabe et al. 2004; Sangiorgi et al. 2006; Karapanagiotidis et al. 2009; Wen et al. 2009
MMP-12	Aortic aneurysms and dissections	Koullias et al. 2004; LeMaire et al. 2005; Karapanagiotidis et al. 2009; Flondell-Site et al. 2010; Didangelos et al. 2011
MMP-13	Aortic aneurysms and dissections	Koullias et al. 2004; LeMaire et al. 2005; Wilson et al. 2004; Karapanagiotidis et al. 2009; Flondell-Site et al. 2010; Didangelos et al. 2011; Saracini et al. 2012
MMP-12	ATS	Matsumoto et al. 1998; Luttun et al. 2004; Morgan et al. 2004; Liang et al. 2006; Yamada et al. 2008; Scholtes et al. 2012
MMP-12	CAD	Jguirim-Souissi et al. 2007
MMP-12	AAA	Curci et al. 1998; Longo et al. 2005
MMP-12	AAD	Del Porto et al. 2010; Song et al. 2013; Proietta et al. 2014; Del Porto et al. 2014

The contribution of MMPs to CVD has been extensively reported (Table 2), whereas their role as biomarkers and prognostic factors is not fully elucidated. Among MMPs, MMP-9 is the most studied in CVD, and its levels have been

associated with several ATS features. In particular, it has been suggested that MMP-9 could be considered as an independent risk factor for cardiovascular mortality in patients with CAD; nevertheless, the association between its plasma levels and cardiovascular risk among patients with CAD is attenuated after adjustment for traditional risk factors such as CRP, IL-6, and fibrinogen (Blankenberg et al. 2003). MMP-2 suffers from a similar limitation. Its plasma levels increase in a wide spectrum of CVD making difficult its use in clinical practice. Differently from the others MMPs, MMP-12 seems to be specifically related to aortic wall weakening diseases. In particular, it has been demonstrated that MMP-12 represents an useful markers distinguishing patients with Stanford-A AAD from those with stable carotid artery stenosis, suggesting that such macrophage elastase is electively released during aortic rupture, in which macrophages have been demonstrated to be the main players. MMP-12 seems also to be involved in promoting plaque instability, despite its usefulness as specific biomarker is weakened by the great number of mediators released by immune cells involved in plaque remodeling.

In conclusion, MMPs are deeply involved in the different stages of atherosclerotic lesion, although further studies are needed to clarify their specific activities in the different features of ATS and to evaluate their application as biomarkers. Nevertheless, among all, MMP-12 seems to be useful in discriminating among patients with the same traditional cardiovascular risk factors, those having a higher risk to develop an aortic disease.

Summary Points

- Atherosclerosis, one of the major causes of cardiovascular diseases, is an inflammatory multifactorial disease, in which the complex interaction between immune cells and inflammatory mediators drives the growth of atherosclerotic lesions and their progression toward complications.
- Among all mediators, metalloproteinases (MMPs) are deeply involved in the pathological process underlying CVD, through their ability to degrade matrix components.
- An interesting specific role has been suggested and confirmed for MMP-12 in the development of Stanford-A acute aortic dissection; indeed, different from the others MMPs, it seems to be specifically related to aortic wall weakening diseases.
- Identification of biomarkers useful to identify patients having a high cardiovascular risk has represented a challenge in the last years.
- The most useful markers are those that appear in the early phases of the disease, that have predictive positive value, and that can help to distinguish between low and high clinical cardiovascular risk.
- Taking into account the great number of molecules and cells involved in ATS, over the time several markers such as pro-inflammatory cytokines and chemokines, acute phase response proteins, blood cells, and proteins implicated in lipid metabolism have been evaluated; however, they did not often represent useful biomarkers in the clinical practice due to their poor specificity.

- Among MMPs, MMP-12 seems to be a useful marker to discriminate among patients with the same traditional cardiovascular risk factors and those having a higher risk to develop an aortic disease.

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Homocysteine as a Biomarker in Vascular Disease

17

Pilar Codoñer-Franch and Eulalia Alonso-Iglesias

Contents

Abstract

Elevated concentrations of homocysteine (Hcy) result from either mutations in the genes encoding Hcy-metabolizing enzymes or from deficiencies of their

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_11

cofactors. Even a mild increase in the levels of Hcy is considered a risk factor for a number of diseases in humans, such as cardiovascular disease, stroke, neurodegenerative disorders like dementia and Alzheimer's disease, birth defects, complicated pregnancies, and bone fractures. However, it has not yet been elucidated whether Hcy is a causative agent. Here, we present an overview of recent data on the putative mechanisms of Hcy in hyperhomocysteinemia-related vascular diseases. However, the mechanism by which Hcy can promote atherogenesis remains unclear. Endothelial dysfunction is the central condition on which a number of factors converge. Increased oxidative stress with alterations in nitric oxide, protein thiolation, and homocysteinylation, as well as Hcy-induced epigenetic changes, are involved in the pathogenesis. Although combined folic acid and B-vitamin therapy substantially reduces Hcy levels, the results are mixed from most clinical trials testing the benefit of vitamin supplementation on cardiovascular events, but they have generally failed to show a significant effect.

Keywords

Cardiovascular disease • Homocysteine • Hyperhomocysteinemia • Risk factor • Thrombosis • Vascular disease

Abbreviations

5-MTHF	5-methyltetrahydrofolate
ADMA	Asymmetric dimethylarginine
CBS	Cystathionine β-synthase
CSE	Cystathionine γ-lyase
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
GSH	Glutathione
Hcy	Homocysteine
HDL	High density lipoprotein
HHcy	Hyperhomocysteinemia
iNOS	Inducible nitric oxide synthase
MAT	Methionine adenosyltransferase
MTHFR	Methylenetetrahydrofolate reductase
MTs	Methyltransferase
NFκB	Nuclear factor –kappa B
NO	Nitric oxide
NOS	Nitric oxide synthase
ROS	Reactive oxygen species
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
tHcy	Total homocysteine

Key Facts of Homocysteine

- Homocysteine is an amino acid that is produced by the body.
- Homocysteine is biosynthesized from the methionine, another amino acid, ingested from the foods.
- In homocysteine metabolism, folic acid, vitamin B12, and vitamin B6 are implicated.
- Homocysteine levels in plasma depend on cellular metabolism of this amino acid.
- A high level of homocysteine in blood can increase the risk for arteriosclerosis.
- A high level of homocysteine has been also implicated in stroke, Alzheimer's disease, or birth defects.
- There are genetic variants involved in an increase in homocysteine levels.
- There is no evidence at this time that supplementation of vitamins and decreased homocysteine levels improve outcomes for cardiovascular risk.

Definitions

Endothelial dysfunction The earliest manifestation of vascular disease. It is characterized by the incapacity of the endothelium to sustain an adequate vasodilator and antithrombotic response to physiological needs. In other words, a shift of the endothelium towards limited vasodilation, proinflammatory and prothrombotic properties.

Folate cycle Set of cyclic reactions implicated in the conversion and recycling of folate into methyltetrahydrofolate (5-MTHF), its active form as a methyl donor.

Homocysteine levels in plasma Refers to the levels of total circulating homocysteine that are conventionally assessed in analytical practice. It includes both free homocysteine and homocysteine bound to other metabolites or molecules through disulfide bonds.

Homocysteine A sulfur-containing amino acid that is not provided by proteins, but is generated as an intermediary metabolite in the conversion of methionine into cysteine.

Hyperhomocysteinemia The term is usually used to refer levels of circulating homocysteine exceeding 15 μM. This cut off value must be contextualized according to age, gender and lifestyle factors.

N-Homocysteinylation A spontaneous non-enzymatic reaction of homocysteine thiolactone with the ε-amino group of lysine residues in proteins to form N-linked Hcy-protein adducts.

Oxidative stress A metabolic imbalance between pro-oxidant and antioxidant species that favor oxidative damage to biological molecules and over activation of redox-sensitive pathways.

Remethylation pathway The conversion of homocysteine back to methionine by transfer of a methyl group that, in most of the tissues, is provided by 5-methyltetrahydrofolate (5-MTHF), in a reaction that is folate- and vitamin B12-dependent.

S-Homocysteinylation A spontaneous non-enzymatic oxidation reaction between the -SH group (thiol group) of homocysteine and the -SH group of cysteine residues in proteins to form S-S (disulfide bond)-linked Hcy-protein adducts.

Transmethylation pathway A series of enzyme catalyzed reactions implicated in the generation of homocysteine from methionine. An intermediate in this process is S-adenosylmethionine, the main provider for methyl groups in biological reactions, such as those implicated in the epigenetic control of gene expression.

Transsulfuration pathway A set of vitamin B6-dependent enzyme catalyzed reactions that account for the conversion of homocysteine into cysteine.

Introduction

Vascular disease is a general term that comprises a class of diseases encompassing several pathological states involving the heart and systemic blood vessels. It results from a complex multicellular and inflammatory response that involves all of the layers of the vessel wall and may lead to obstructed blood flow. Arteriosclerotic vascular disease, also known as atherosclerosis, is marked by the atherosclerotic plaque, which contains lipids, inflammatory cells, smooth muscle cells, connective tissue, and calcium deposits (Van Campenhout et al. 2009). Therefore, the mechanism of atherosclerosis involves several highly interrelated processes, such as lipid disturbances, oxidative stress and inflammation that imply endothelial dysfunction, vascular smooth cell activation, altered matrix metabolism, increased platelet activation, and thrombosis. The stiffened and narrowed blood vessels with a thrombus limit blood circulation, which threatens the functionality of the main vital organs, such as the heart and brain. Several pathological conditions can then arise, primarily including cardiovascular and peripheral vascular diseases, cerebrovascular events, and neurodegenerative diseases. In this sense, vascular disease is the leading cause of death and disability in developed nations and is rapidly increasing in the developing world (Wong 2014).

Risk factors play an important role in initiating and accelerating the complex process of atherosclerosis. Aside from nonmodifiable risk factors, such as age, gender, and family history/genetics, the major modifiable risk factors include hypertension, dyslipidemia, smoking, obesity, and diabetes mellitus. A number of

emerging risk factors for atherosclerosis have recently been proposed to help identify high-risk individuals (Jensen et al. 2014). Among these newer risk factors, elevated plasma levels of the sulfur-containing amino acid, homocysteine (Hcy), has received a great deal of interest in the past few years, primarily due to its prevalence in the general population.

However, to consider Hcy as a causal risk factor for vascular disease, three requirements should be fulfilled: (1) identification of the mechanisms by which Hcy promotes atherothrombosis; (2) demonstration of a significant association between elevated Hcy levels and adverse vascular outcomes in epidemiological studies; and (3) risk modification by reducing Hcy levels. Elevated Hcy concentrations were identified as a potentially modifiable risk factor for abdominal aortic aneurysm (Cao et al. 2014), stroke (He et al. 2014), coronary events (Schaffer et al. 2014), venous thromboembolism (Cohoon and Heit 2014), and death (Jung et al. 2013). However, there is controversy as to whether Hcy is a pathological cause or merely a marker, because several intervention trials have failed to demonstrate any clinical benefit from Hcy-lowering therapy (Toole et al. 2004; Lonn et al. 2006). Although there is consensus that hyperhomocysteinemia (HHcy) is associated with several vascular pathological conditions, the mechanisms by which elevated Hcy impairs vascular function are not completely understood. In the present chapter we will discuss the role of Hcy in mediating vascular dysfunction and whether it leads to an increased risk of atherothrombosis.

Biochemistry

Hcy is a sulfur-containing nonproteic amino acid that was discovered in 1932 by Du Vigneaud (Nobel Prize in Chemistry in 1955) and is produced by the conversion of methionine, an essential amino acid present in foods and regularly consumed within the diet. It is an intermediate metabolic product, leading to cysteine biosynthesis from methionine. Although Hcy is a normal metabolite involved in fundamental biological processes, excess Hcy can be extremely toxic. Therefore, its metabolism is highly regulated (Selhub 1999; Stipanuk 2004).

Homocysteine Metabolism

The Methionine-Homocysteine Transmethylation Pathway

The first step in Hcy biosynthesis is the reaction between methionine that is absorbed from the digestive system and ATP to generate S-adenosylmethionine (SAM), which is catalyzed by methionine adenosyltransferase (MAT) (Fig. 1). SAM is an activating compound that acts as the main biological donor of methyl groups in humans, and the liver is the principal organ implicated in its synthesis and utilization. This is a consequence of the high levels of liver-specific expression of the MAT1A-gene that encodes for the catalytic subunits of the MATI/III isoenzymes. The MAT1A-encoded MATIII isoform is the predominant isoenzyme in liver and is characterized

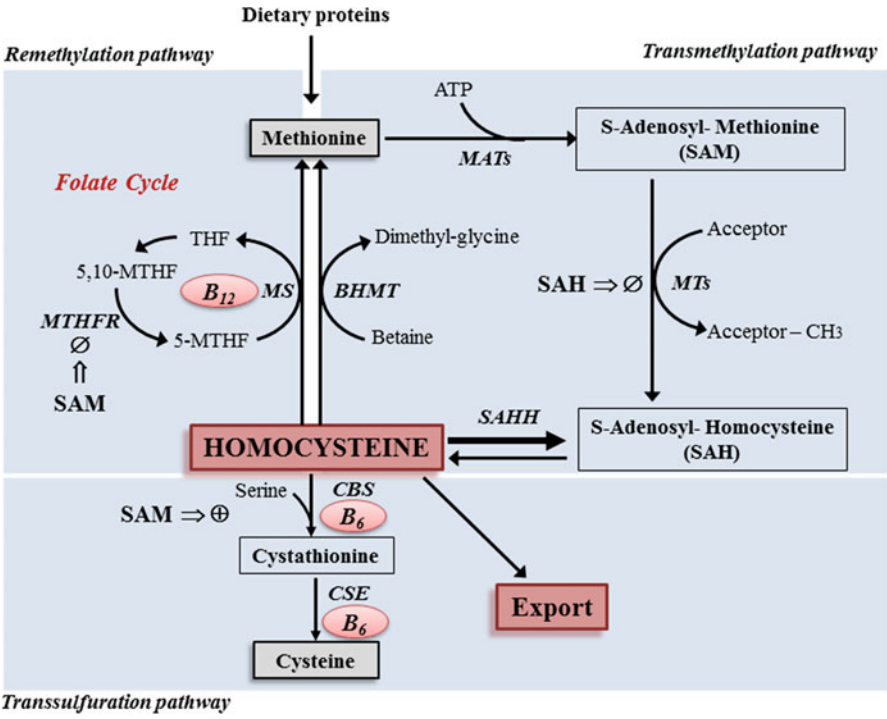

Fig. 1 Homocysteine is at the cross roads of the transmethylation, remethylation and transsulfuration pathways that connect methionine and cysteine metabolism. Remethylation of homocysteine back to methionine using betaine as the methyl donor is restricted to liver. B_{12}, vitamin B_{12}; B_6, vitamin B_6; BHMT, betaine homocysteine methyltransferase; CBS, cystathionine β-synthase; CSE, cystathionase (cystathionine γ-lyase); MATs, methionine adenosyltransferase isoenzymes; MS, methionine synthase; MTHFR, 5,10-methylenetetrahydrofolate reductase; MTs, methyltransferases; SAH, S-adenosylhomocysteine; SAHH, S-adenosylhomocysteine hydrolase; SAM, S-adenosylmethionine; THF, tetrahydrofolate; 5,10-MTHF, 5,10-methylenetetrahydrofolate; 5-MTHF, 5-methyltetrahydrofolate. Enzyme inhibition (∅) and activation (⊕) by SAM and SAH are indicated

by a high Km value for methionine (approximately 200 μM) and positive cooperative regulation by SAM, the product of its reaction. This confers to the liver a proper metabolic response to methionine loads allowing the derivation of the excess to SAM biosynthesis. This is opposite to the situation in extrahepatic tissues where the expression of MAT is restricted to the MATII (MAT2A-encoded) isoenzyme, with a low Km for methionine (approximately 8 μM) and feedback inhibition by SAM (Stipanuk 2004; Ramani et al. 2011; Lu and Mato 2012).

The transference of the methyl group of SAM to different and specific acceptor molecules takes place in the next step of the transmethylation pathway and is catalyzed by methyltransferases (MTs). Common nucleophilic recipients of the methyl group of SAM are carbon, oxygen, nitrogen, and sulfur atoms on nucleic acids, proteins, and other high or low molecular weight molecules and metabolites.

To date, the number of members of the SAM-dependent MTs group is not known, but, in practice, all methylation reactions are SAM dependent, with the remarkable exception of those involved in Hcy remethylation to methionine. The decarboxylation of SAM by SAM-decarboxylase is a highly regulated and critical step in the biosynthesis of polyamines, ubiquitous metabolites related to cell growth, proliferation, and differentiation, not only in liver but also in all tissues. Finally, the activities of the SAM-dependent DNA MTs and histone MTs are relevant to the epigenetic control of the flux of the genetic information, a process potentially implicated in relating HHcy to cardiovascular risk (Lu and Mato 2012).

Through the transference of its methyl group, SAM is converted into S-adenosylhomocysteine (SAH), an obligatory coproduct and a potent competitive inhibitor of MTs activity. Thus, the level of SAM and the ratio SAM/SAH will be relevant in determining MTs activity and the extent of specific transmethylation reactions (Wagner and Koury 2007). The hydrolysis of SAH by SAH hydrolase reduces SAH levels, producing Hcy and adenosine. However, the reaction catalyzed by SAH hydrolase is reversible and its equilibrium constant greatly favors SAH synthesis under physiological conditions. The consequences of the inhibitory effect of SAH on MTs activity and the reversibility of the exchange reaction between Hcy and SAH must be considered in HHcy. In any case, it is necessary to remove the Hcy and adenosine products to reduce and sustain low SAH levels. This is achieved by a combination of regulated Hcy export out of the cells and intracellular processing of Hcy through two key processes: (1) remethylation in the methionine cycle that synthesizes methionine from Hcy utilizing 5-methyltetrahydrofolate (5-MTHF) or betaine (in liver and kidney) as methyl donors and links to the folate cycle and (2) irreversible transsulfuration that converts Hcy to cystathionine and eventually to cysteine (Selhub 1999; Stipanuk 2004).

Remethylation Pathway

The remethylation pathway ensures the recycling of Hcy to methionine. In practically all tissues, Hcy remethylation is catalyzed by methionine synthase (MS), a vitamin B12-dependent folate enzyme that uses 5-MTHF as the methyl group donor. The products of the MS catalyzed reaction are methionine and tetrahydrofolate, which must be reconverted to the active form of 5-MTHF through the folate cycle. In the course of this cycle, the final regulatory step is the irreversible reduction of 5,10-methylenetetrahydrofolate to 5-MTHF by methylenetetrahydrofolate reductase (MTHFR), a flavoenzyme that uses NADH as the electron donor and is negatively regulated by SAM. In the liver, an additional pathway for Hcy remethylation to methionine implicates betaine-Hcy MT, which uses betaine (a choline-derived metabolite) as the methyl donor instead of 5-MTHF and is not vitamin B12 dependent. The remethylation of Hcy by betaine is relevant to ensuring methionine recycling under conditions in which the folate pathway is impaired (Blom and Smulders 2011).

Transsulfuration Pathway

In the transsulfuration pathway, Hcy is unidirectionally converted to cysteine through the consecutive reactions catalyzed by cystathionine β-synthase (CBS)

and cystathionase (cystathionine γ-lyase; CSE), two enzymes requiring the active form of vitamin B6, pyridoxal 5-phosphate. In this reaction, the sulfur atom of Hcy is transferred to serine and retained in the cysteine molecule, whereas the remaining Hcy carbon backbone is released as α-ketobutyrate and exported. Therefore, the transsulfuration pathway is limited to tissues that coexpress both enzymes, such as the liver, where cysteine synthesis from Hcy may be particularly relevant to sustain its high rate of protein synthesis and glutathione (GSH, γ-glutamyl-cysteinyl-glycine) demands. GSH is the most abundant intracellular low molecular weight thiol, and the liver is its main producer, where cysteine availability is a limiting factor for its synthesis. Among its many other functions, GSH plays an important role in antioxidant defense as the major intracellular redox buffer. A reduction in GSH levels is a hallmark of oxidative stress associated with many physiological and pathological states (Wu et al. 2004). Several cellular processes, such as proliferation, apoptosis, and macromolecular synthesis functions, are regulated by redox signaling. Redox sensing of CBS will contribute to adapting the flux of Hcy through the transsulfuration pathway to the demand of cysteine for GSH synthesis during oxidative stress. In addition, the two enzymes of the transsulfuration pathway, CBS and CSE, are also implicated in several reactions between cysteine and Hcy that generate hydrogen sulfide (H_2S), a potent antioxidant and signaling molecule that causes vasodilation. The inhibition of CSE by homocysteinylation and reduced production of H_2S have been proposed, among others, as molecular links between HHcy and its associated hypertension and vascular disease (Sen et al. 2010).

Under physiological conditions and adequate vitamin supply, the main determinant of Hcy flux is methionine intake, and the liver is the center of the metabolic response. The liver is able to keep Hcy at adequate levels by switching its flux through the remethylation and transsulfuration pathways, depending on methionine supply. When the capacity of transsulfuration pathway is exceeded, Hcy is exported from the cell. To maintain sulfur balance, the total intake of methionine and cysteine should equal elimination, primarily as sulfate and taurine. Hence, the methionine-Hcy cycle and its associated pathways are crucial to several important biological reactions, such as methylation and redox balance, thereby involved in nucleic acid synthesis and also sulfur balance (Stipanuk 2004; Sen et al. 2010).

Hcy is also metabolized to its reactive anhydride cyclic thioester, Hcy-thiolactone, by methionyl-tRNA synthetase. Hcy-thiolactone is cytotoxic to the cardiovascular system. The Hcy-thiolactone pathway predominates when remethylation or transsulfuration reactions are impaired by genetic alterations of the enzymes involved in Hcy metabolism or by an inadequate supply of folate, vitamin B12, or vitamin B6 (Jakubowski 2008; Perla-Kaján and Jakubowski 2012).

Circulating Homocysteine

The export of Hcy from the cells into the circulation contributes to maintaining it at a low intracellular level and, in the absence of kidney malfunction, circulating Hcy is

considered to reflect the balance of the intracellular Hcy metabolism. The condition of HHcy arises from disturbances of Hcy metabolism that promote its accumulation in the cells and subsequent disposal in the blood. This may be triggered by genetic defects of the enzymes of Hcy metabolism or, more frequently, by nutritional deficiencies of the vitamins required for satisfactory Hcy processing (folic acid, B12, B6), inadequate methionine intake, or other factors. The severity of HHcy will be dependent on its metabolic origin. The intake of a large, protein-rich meal may increase the plasma Hcy concentration by 10–15 % after 6–8 h (Refsum et al. 2004).

Levels of Homocysteine

There are no internationally accepted reference values for plasma Hcy. Several reasons can account for this variability, such as the status of the enzymes involved in methionine metabolism and the levels of their cofactors, folic acid and vitamins B12 and B6. The status of these cofactors is strongly and inversely correlated with plasma Hcy levels. Hence, it is difficult to define an apparently safe level when the vitamin status is not known. Additionally, the values increase with age. The most accepted Hcy plasma levels in normal adults range between 5 and 15 μM. Fasting levels of Hcy of greater than 15 μM will be considered as HHcy and classified as moderate (16–30 μM), intermediate (31–100 μM), or severe (>100 μM) (Refsum et al. 2004) (Table 1). Severe HHcy and homocystinuria may be caused by deficiency of either homozygote CBS, homozygote thermostable MTHFR, or the enzymes catalyzing vitamin B12 metabolism. Mild or moderate HHcy usually reflects an impaired remethylation pathway. The possible causes include folic acid or vitamin B12 deficiency or MTHFR dysfunction. A common point mutation, 677C→T, in the MTHFR gene causes an alanine-to-valine substitution and is associated with reduced MTHFR enzyme activity. Individuals with the MTHFR 677TT genotype usually have 2.5 μmol/L higher tHcy than those with the 677CC variant, but this depends on the folate and riboflavin status. Hcy levels in the moderate range can be a consequence of defective Hcy transsulfuration due to vitamin B6 deficiency or impaired renal function. Abnormal increases of plasma Hcy (>15 μmol/L) after a methionine load (100 mg/kg) may reflect impaired Hcy transsulfuration due to deficiency of heterozygous CBS or vitamin B6.

Unless specified, plasma levels of Hcy used in clinical practice refer to the sum of all Hcy circulating species, which is termed *total Hcy* (tHcy) (Refsum et al. 2004). In plasma, the free reduced form of Hcy represents a minimal fraction (~1 %). The bulk of Hcy is conjugated with proteins (80–85 %; principally albumin) or low molecular weight thiols (15–20 %), mainly with cysteine and other Hcy molecules (homocystine). In practice, this problem has been solved by treating plasma (or serum) with a reductant prior to Hcy determination. This treatment cleaves the disulfide bonds and converts all Hcy species into the free Hcy form, allowing for its analytical determination as tHcy. At present, clinical testing for tHcy has been very much improved with the introduction of enzyme immunoassays adapted for automated platforms. This method shows acceptable performance and is comparable to the classical enzymatic or HPLC methodologies (La'ulu et al. 2008).

Table 1 Classification and the most common causes of fasting homocysteine level increase

Classification - level	Causes
Normal (5–15 μM)	Adequate lifestyle factors
	Low levels in this range are associated with low risk of vascular disease
Moderate (16–30 μM)	Moderate folate or B_{12} deficiency
	B_6 deficiency
	Genetic predisposition (MTHFR C677T)
	Initial/moderate kidney disease
	Hepatic dysfunction
	Age: levels rise with increasing age; menopause
	Sex: levels higher in males
	Inadequate lifestyle factors: excessive coffee or alcohol consumption; smoking; reduced physical activity; dietary excess of methionine-rich animal proteins
	Drugs: folate, B_6 or B_{12} antagonists; anticonvulsants; contraceptives; immunosupressive drugs; some antidiabetic and lipid-lowering treatments
	Diseases: summarized in Table 2
Intermediate (31–100 μM)	Severe folate deficiency or moderate folate or B_{12} deficiency in genetically predisposed populations (MTHFR C677T; MTHFR or CBS heterozygosity)
	Advanced/chronic renal insufficiency
	Diseases: summarized in Table 2
Severe (>100 μM)	Genetic severe deficiencies in CBS, MTHFR, MS or enzymes related to B_{12} metabolism
	Severe B_{12} deficiency

CBS cystathionine β-synthase, *MS* methionine synthase, *MTHFR* 5,10-methylenetetrahydrofolate reductase

Factors that influence tHcy should be taken into account when interpreting tHcy results. Studies on tHcy should consider the common and important genetic MTHFR 677CT polymorphism, physiological factors (age, sex, pregnancy, menopausal state, renal function), lifestyle (smoking, coffee intake, diet, drugs), and cofactors (folate, cobalamin) as determinants of tHcy levels.

Mechanisms Involving Homocysteine in Atherothrombosis

History

The link between HHcy and atherothrombotic cardiovascular disease was originally proposed as the "homocysteine theory of arteriosclerosis" more than 40 years ago by McCully, who observed advanced arterial lesions in children with homocystinuria, an inborn error in methionine metabolism that causes extremely high serum Hcy

levels (>100 μM) (reviewed in McCully 2007). The key finding was that 50 % of homocystinuric children died prematurely from vascular diseases. The first solid evidence of the relationship between Hcy and cardiovascular disease in the general population was provided in 1976, when Wilcken and Wilcken showed that patients with coronary artery disease suffered from an abnormal Hcy metabolism more often than controls. Since then, the association of HHcy with cardiovascular disease has long been established (Malinow 2001). Nevertheless, it is a subject of debate whether moderate increases in serum Hcy cause cardiovascular disease. Abundant epidemiological studies provided evidence that elevated blood Hcy concentrations confer an independent increased risk for atherosclerotic disease (Lentz 2005), and numerous experimental and clinical studies have addressed this interesting link. Moderate HHcy is currently considered as an independent risk factor for cardiovascular disease and is responsible for approximately 10 % of total risk. It is positively associated with the risk of deep vein thrombosis, pulmonary embolism, and stroke (Wald et al. 2002). HHcy is also associated with other several pathological conditions, such as diabetes, neurodegenerative diseases, osteoporosis, and birth defects (Table 2) (Hooshmand et al. 2013; Enneman et al. 2014; Iacobazzi et al. 2014). However, four decades later, it has not been conclusively confirmed whether moderate HHcy is a causative factor or a biomarker of cardiovascular disease.

Table 2 Diseases related to homocysteine increase

Cardiovascular diseases	Neurodegenerative diseases
Myocardial infarction Brain stroke Ischemic heart disease Atherosclerotic vessel damage Peripheral arterial occlusive disease Venous thrombosis Hypertension Hemostasis defects	Parkinson's disease Depression Dementia Cognitive defects Schizophrenia Multiple sclerosis Cognitive decline of the elderly
Pregnancy and birth defects Neural tube defects Spina bifida Reproductive function Pregnancy complications Abortion Other congenital defects: cleft lip/palate, anencephalies, heart defects	***Gastrointestinal disorders*** Mesenteric venous thrombosis Bowel infarction Inflammatory bowel disease Crohn's disease Colorectal cancer
Autoimmune diseases Rheumatoid arthritis Lupus erythematosus Diabetes (related to nephropathy) Immune activation related to inflammation	***Other diseases/conditions*** Cancer/ hyperproliferative disorders Psoriasis Osteoporosis Hypothyroidism Hepatic dysfunction Wound healing Sickle-cell anemia

Pathophysiology

The mechanisms by which elevated Hcy levels might contribute to atherogenesis and thrombosis are incompletely understood and multiple hypotheses to explain the induced pathophysiology are being studied. Several of them, including protein homocysteinylation and oxidative stress, are directly triggered by Hcy. Other metabolites generated by Hcy can be responsible of the toxic effects (Fig. 2). SAH, the precursor of Hcy in the methionine metabolic cycle, has recently emerged as a more sensitive indicator of cardiovascular disease than Hcy (Xiao et al. 2013). The accumulation of SAH leads to the disruption of the methylation cycle, modification of the SAH/SAM ratio and methylation deficiency, an important mechanism involved in Hcy pathology. The extent of damage induced by Hcy or related metabolites may be determined by diverse factors, such as tissue composition, Hcy uptake, intermediate metabolism, and the duration of Hcy exposure.

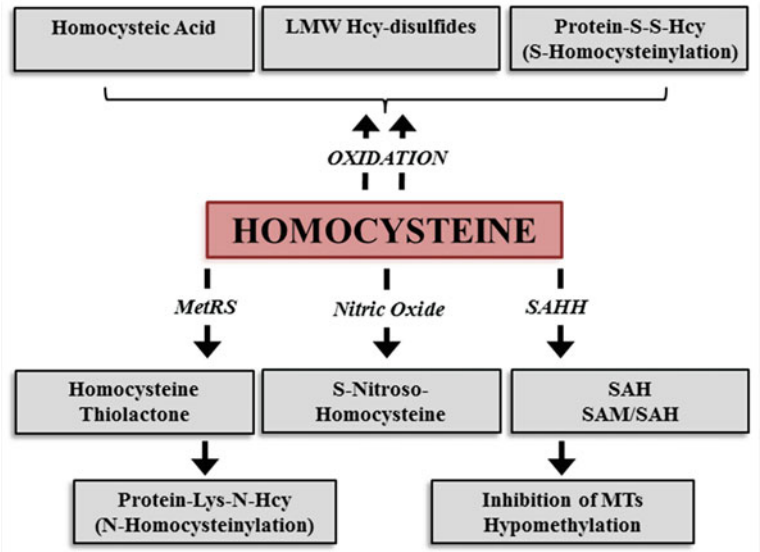

Fig. 2 Metabolites related to direct and indirect toxic effects of homocysteine. The spontaneous oxidation of the SH group of homocysteine accounts for the generation of homocysteic acid, low molecular weight (LMW) homocysteine disulfides (Hcy-S-S-Hcy (homocystine) and Hcy-S-S-Cys), and Homocysteine attachment to cysteine residues in proteins through disulfide bridges (S-Homocysteinylation). The conversion of Homocysteine to its reactive intramolecular thioester thiolactone form is catalyzed by methionyl-tRNA synthetase (MetRS), and accounts for the generation of N-linked Hcy-protein adducts (N-Homocysteinylation) that disturb protein structure and functionality and elicit autoimmune responses. S-Nitroso-homocysteine is generated from the reaction between Homocysteine and nitric oxide. The equilibrium of the S-adenosylhomocysteine hydrolase (SAHH) reaction may be shifted towards the formation of S-adenosylhomocysteine (SAH) under conditions of excess homocysteine. SAH is a potent inhibitor of most S-adenosylmethionine-dependent methyl transferases (SAM-MTs), whose activity will be affected by the SAM/SAH ratio

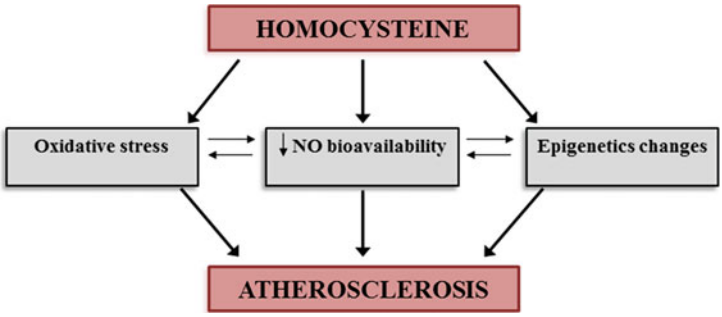

Fig. 3 Proposed mechanisms for hyperhomocysteinemia effects on vascular diseases. Hyperhomocysteinemia generates oxidative stress, reduces nitric oxide bioavailability and induces epigenetic changes mainly through hypomethylation

It is now widely accepted that Hcy exerts a detrimental effect on the vascular wall, particularly on endothelial cells, by increasing intracellular oxidative stress and decreasing NO bioavailability and by triggering multiple proatherogenic mechanisms through epigenetic changes (Fig. 3). Observations in clinical and animal studies have identified potential pathophysiological targets on which Hcy exerts its damaging effect. Those targets include endothelial cells, nitric oxide (NO) signal transduction molecules, redox-sensitive inflammatory pathways, low-density lipoproteins, smooth muscle vascular cells, and the coagulation system, among others. Some of these mechanisms are summarized and addressed below (Fig. 4).

Endothelial Dysfunction

Endothelial dysfunction, defined as the impairment of normal homoeostatic properties of the vascular endothelium, is a key event in vascular pathology and the development of vascular diseases. It results in decreased vasodilator capacity, vascular remodeling, and inflammation through increased recruitment of circulating leukocytes to adhere to the endothelium and differentiate into macrophages. This leads to the primary injury in the progression of atherosclerotic lesions. All of these mechanisms have been associated with elevated plasma levels of Hcy. Hcy exerts a detrimental effect on the vascular wall, particularly on endothelial cells, and triggers multiple proatherogenic mechanisms.

Homocysteine and Oxidative Stress

Direct Hcy-mediated endothelial cell damage and endothelium dysfunction has been related mainly to an increase in oxidative stress (Papatheodorou and Weiss 2007), a very basic biological process that underlies a variety of pathologies. It is initiated by the generation of potent reactive oxygen species (ROS). Hcy contains a reactive sulfhydryl group (−SH, thiol) and, such as most thiols, has the ability to oxidize in the presence of an electron acceptor, such as molecular oxygen, at physiological pH. The thiol oxidation reaction is favored by transition metals such as copper. This

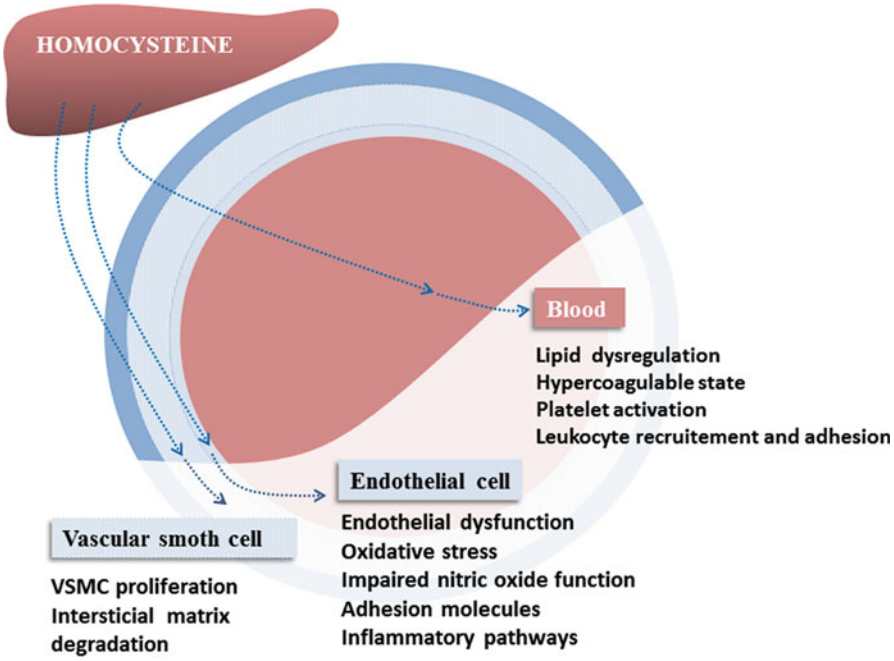

Fig. 4 Potential pro-atherogenic effects of hyperhomocysteinemia (HHcy) at the vascular level. In the circulation, HHcy promotes the dysregulation of circulating lipids by LDL oxidation and reduces HDL-cholesterol levels by inhibiting the hepatic synthesis of apoprotein A-I, which may be mediated by endoplasmic reticulum stress. HHcy enhances a thrombotic state by producing a hypercoagulable state and platelet activation and aggregation. Additionally, supra-physiological levels of homocysteine (Hcy) upregulate chemokines and adhesion molecules to enhance the responsiveness of leukocytes, leading to increased leukocyte recruitment and adhesion to vascular wall. In the vessel wall, Hcy induces endothelial cell dysfunction, impairs nitric oxide endothelium-dependent vasodilator function and stimulates several inflammatory pathways by the activation of the redox-sensitive transcription factor NF-kB. In addition, Hcy stimulates vascular smooth muscle cell proliferation and proteolysis of the extracellular matrix

auto-oxidation of Hcy leads to the generation of disulfides and potent ROS species such as superoxide and hydrogen peroxide. The main oxidized disulfide forms were the Hcy homodimer *homocystine*, the *Hcy-cysteine* and the *Hcy-GSH mixed disulfides*, and the disulfides resulting from the linkage of Hcy to the -SH groups of cysteine residues in plasma proteins (S-homocysteinylation). These compounds are referred to as oxidized forms of Hcy in plasma and arise primarily through disulfide exchange, with only a small fraction resulting from direct Hcy oxidation.

In addition, Hcy has been shown to inhibit intracellular antioxidant enzymes, including glutathione peroxidase and superoxide dismutase, thus decreasing the cell's ability to neutralize oxidant radicals. Another mechanism of cytotoxicity may involve the inactivation of the GSH antioxidant defense system by limiting GSH synthesis. Hcy can also increase the activity of the pro-oxidant enzyme NADPH oxidase (Becker et al. 2005).

Alterations of Hcy levels may cause an imbalance between the production of ROS and the antioxidant defenses that were insufficient to counteract them, resulting in the initiation of lipid peroxidation and subsequent protein and nucleic acid damage. Cellular dysfunction, and occasionally cell death, then arises with deleterious effects on the functional and structural integrity of biological tissues. In this way, Hcy can have a detrimental effect on vascular cells, starting a process leading to the disruption of endothelial function.

Noticeably, vascular endothelial cells are very sensitive to even a mild increase in Hcy concentration. This susceptibility may be explained by the fact that the human endothelial cells do not express the active form of CBS, and, consequently, they are not able to initiate Hcy catabolism via the transsulfuration pathway. Increased vascular oxidant stress in HHcy leads to a decrease in (1) the bioavailability of the signaling molecule nitric oxide and (2) upregulation of redox-sensitive proinflammatory signaling pathways in vascular cells.

Homocysteine and Nitric Oxide

NO is a molecule with an important regulatory role in a variety of biological functions. NO is synthesized by NO synthase (NOS; EC 1.14.13.39), with three isoforms: two constitutive calcium/calmodulin-dependent isoforms, neuronal NOS (nNOS, NOS I) and endothelial NOS (eNOS, NOS-III); and an inducible calcium-independent NOS (iNOS, NOS-II), which is expressed in macrophages and inflammatory cells (Moncada and Higgs 2006). NO is the main vasodilator of the organism and is crucial for maintaining proper vascular tone. NO released toward the vascular lumen is a potent inhibitor of platelet aggregation and adhesion. Hence, a decrease in NO availability may have a dramatic impact on vascular function. The molecule is habitually synthesized in the endothelial lining of blood vessels by eNOS (Becker et al. 2005). It has been shown that Hcy impairs endothelium-dependent vasodilator function, mainly due to a decrease in bioavailable NO. Furthermore, Hcy has been associated with a reduction in NO synthesis by endothelial cells and also produces endogenous NO inhibitors.

Nitric Oxide Synthesis Reduction

Hcy may reduce the amount of NO via several mechanisms. First, persisting oxidative stress in HHcy will render eNOS dysfunctional. This altered enzyme no longer produces NO but increases ROS generation. Second, Hcy induces NADPH oxidase and iNOS, which both contribute to increased ROS production. Third, Hcy may favor eNOS inhibition by increasing the levels of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of eNOs. ADMA is generated during the degradation of proteins containing methylated L-arginine residues, and its disposal is very sensitive to oxidative stress. In addition, Hcy has been shown to inhibit arginine transport in endothelial cells. The increase in the ADMA/arginine ratio not only results in the inhibition of eNOs (Stühlinger et al. 2001), but it also leads to a lower availability of L-arginine for the formation of NO. Alternatively, Hcy may promote the oxidation of tetrahydrobiopterin, an obligatory cofactor for eNOS, thus favoring uncoupling of the enzyme and ROS generation (He et al. 2010).

Inhibition of Nitric Oxide

Oxidative inactivation of NO to reduce its bioactivity as a vasodilator is a major mechanism for altered vascular function. It can arise because oxygen radicals react with NO to form peroxynitrite radicals. In conditions of oxidative stress, such as in HHcy, superoxide anions generated in endothelial cells quickly react with endothelium-derived NO to form peroxynitrite. Peroxynitrite can spontaneously and rapidly (a halftime of seconds) isomerize to nitrate, which is eliminated by the kidneys. However, it is also a strong oxidant, causing the oxidation of thiols (e.g., glutathione or protein thiols). It can also promote protein tyrosine nitration by adding nitrate to cellular tyrosine, resulting in nitrotyrosine residues. Furthermore, peroxynitrite initiates a lipid peroxidation chain reaction and limits NO signaling.

In addition, dysfunctional endothelium releases endothelin-1, which, along with decreased NO release, leads to vasoconstriction. Hcy not only reduces the bioavailability of NO but also causes the deterioration of the elastic structure of the arterial wall through alterations of metalloproteinase activity. It may also increase vascular rigidity by augmenting the breakdown of elastin in vascular cells (Steed et al. 2010).

Homocysteine and Inflammation

HHcy has been shown to initiate and/or aggravate inflammation and cytokine production. This may be promoted by a decrease in NO signaling and/or increased vascular oxidant stress. The pro-oxidative state in HHcy has the potential to upregulate redox-sensitive proinflammatory signaling pathways in vascular cells, such as the transcription factor nuclear factor-kappaB (NF-κB). Oxidative stress is involved in the activation of this pathway through the phosphorylation and degradation of the inhibitory protein IκBα. Activated NF-κB is then translocated to the nucleus, where it binds to the enhancer element of many proinflammatory genes and increases the expression of inflammatory mediators.

In this way, HHcy has been shown to activate proinflammatory signaling pathways in endothelial cells (Pushpakumar et al. 2014) and macrophages (Gao et al. 2014). Hcy is able to initiate an inflammatory response mediated through a NMDAR-ROS-ERK1/2/p38-NF-κB signaling pathway, inducing the expression of C-reactive protein and proliferation of vascular smooth muscle cells (Pang et al. 2014).

Moreover, the biochemical cascade triggered by the activation of NF-kB results in elevated levels of proinflammatory cytokines in the circulation, which also take part in the activation of inflammatory processes inside the vascular wall.

Likewise, Hcy has been shown to induce the mRNA and protein expression of the proinflammatory cytokines monocyte chemoattractant protein-1 and interleukin-8 in cultured human aortic endothelial cells (Poddar et al. 2001) and monocytes (Zeng et al. 2004). Furthermore, Hcy has been shown to induce the expression of endothelial adhesion molecules, including intercellular adhesion molecule 1, vascular cellular adhesion molecule 1, P-selectin, and E-selectin, both in vitro and in vivo (Wang et al. 2002).

Homocysteine and Smooth Muscle Function

Hcy may initiate vascular intimal smooth muscle proliferation through the liberation of multiple growth factors by injured endothelial cells. Noteworthy, Hcy upregulates platelet-derived growth factors, which are strong mitogens for vascular smooth muscle cells via DNA methylation in endothelial cells (Zhang et al. 2012).

By affecting smooth muscle cells, Hcy produces connective tissue changes that cause fibrosis, calcification, proteoglycan deposition, and damage to elastic tissue layers with subsequent alteration of the extracellular matrix. Thus, Hcy leads to intimal thickening and elastic lamina disruption. Subsequent calcification and accelerated osteogenic cell differentiation can then occur (Van Campenhout et al. 2009). The process may be directly stimulated by Hcy or may be secondary to the mitogenic effect of endothelial and/or platelet-derived growth factors released by Hcy-induced endothelial cell damage.

Homocysteine and Vascular Thrombosis

The oxidative injury of endothelium in HHcy, combined with the lack of vasculoprotective effects of NO, predisposes the endothelium to thrombotic events. In addition, in vitro studies provide a biochemical background for a hypercoagulation state in HHcy. Hcy is a potent procoagulant that promotes the expression of clotting factors II, V, X, and XII and reduces the activation of protein C and antithrombin III. Therefore, it increases prothrombin activation with deposition of fibrin and mural thrombosis in the artery walls while interfering with the fibrinolytic system (Di Minno et al. 2010). Hcy-mediated oxidant stress has been shown to trigger platelet activation, leading to elevation of soluble CD40 ligand in plasma (Prontera et al. 2007), which, in turn, favors a tendency to thrombosis. Elevated Hcy increases the generation of some isoprostanes from arachidonic acid oxidation that are also able to cause platelet activation and enhanced biosynthesis of thromboxanes, which links in vivo oxidative stress and the risk of thrombosis (d'Emmanuele diVilla Bianca et al. 2013).

After exposure to Hcy, endothelial cells show a procoagulant phenotype, likely by the induction of tissue factor, inhibition of the expression of thrombomodulin, and by enhancing the activation of tissue plasminogen (Zhu et al. 2012) among others.

Role of the Unfolded Protein Response in Hyperhomocysteinemia

Endoplasmic reticulum (ER) stress and the unfolded protein response are also one proposed Hcy toxicity mechanism. The ER plays a pivotal role in proper assisted protein folding and posttranslational modifications. Unfolded protein response failure and protein degradation eventually leads to altered secretion, with toxic accumulation of proteins in the ER lumen. HHcy was shown to induce ER stress (Li et al. 2013), but it is not precisely known how Hcy causes ER stress (Zhang et al. 2001). A number of studies have suggested that Hcy triggers ER stress in endothelial cells (Li et al. 2011). Another possibility is that Hcy-thyolactone is implicated because it causes protein N-homocysteinylation in the ER, leading to damage to secretory proteins (Jakubowski and Głowacki 2011; Yilmaz 2012).

Furthermore, there is a strong correlation between lipid metabolism, the ER stress response, and elevated Hcy levels. Hcy may play a direct role in triglyceride activation and cholesterol biosynthesis by inducing the overexpression of the sterol regulatory element-binding protein, an ER-membrane bound transcription factor that activates the expression of genes encoding key enzymes in cholesterol and triglycerides metabolism and uptake (Werstuck et al. 2001). Decreased high-density lipoprotein cholesterol (HDL) in HHcy is caused in part by decreased hepatic expression of apoprotein A-I (Mikael et al. 2006). In addition, N-homocysteinylation of HDL may reduce the activity of the paraxonase, a multifunctional enzyme with antioxidant capacity, impairing the antiatherogenic function of this lipoprotein (Holven et al. 2008). In turn, paraoxonase has the ability to detoxify the Hcy-thiolactone (Perla-Kaján and Jakubowski 2012).

Homocysteine: A Biomarker of the Link Between Epigenetic Modifications and Vascular Disease

Epigenetic modifications of DNA and chromatin without alterations in the DNA sequence provide a mechanistic link between environmental factors, nutrition, and disease. Epigenetic mechanisms, such as DNA methylation, post-translational modification of histone proteins, and microRNA biogenesis, regulate patterns of gene expression by altering DNA accessibility and chromatin structure. Thus, environmental challenges can influence the risk for cardiovascular disease through the modifications of implicated genes. A variety of evidence has indicated that epigenetic changes play an important role in atherogenesis (Baccarelli and Ghosh 2012). Because Hcy plays a crucial role in SAM methyl-donor levels, plasma Hcy levels may exert their actions through an epigenetic mechanism involving methylation reactions (Zhou et al. 2014).

Homocysteine and Epigenetic Changes

There are three major epigenetic modifications that could be most directly implicated in the epigenetic mechanisms relating Hcy to cardiovascular disease risk: (1) DNA methylation, (2) histone modification, and (3) noncoding RNA regulation.

DNA Methylation

Experimental animal models have demonstrated that DNA methylation has critical roles in the development of atherosclerosis and cardiovascular disease. A family of DNA MTs is involved in both de novo DNA methylation and its maintenance. These enzymes produce the covalent attachment of a methyl group from SAM to the 5' position of a cytosine nucleotide linked to a guanine nucleotide (CpG dinucleotide; "CpG islands"). The presence of a methyl group at a specific CpG dinucleotide site may directly inhibit transcription factor binding, producing transcriptional repression. In this manner, CpG methylation is an important mechanism to ensure transcriptional gene silencing. Patterns of DNA methylation are tightly regulated in a tissue-specific manner, resulting in epigenetic specification of gene expression. In general, DNA methylation is associated with low gene activity (Blattler and

Farnham 2013). However, regions with a high CpG dinucleotide content are located in the regulatory regions of many genes, including promoters and enhancers. Due to their presence in these regulatory regions, changes in the methylation status can either facilitate (hypomethylation) or inhibit (hypermethylation) the expression of a gene. We can speculate that the proatherogenic genes might be hypomethylated to gain more activity, whereas antiatherogenic genes could be hypermethylated along with a loss of protective function. HHcy may, therefore, initiate or promote atherogenesis by modifying DNA methylation. Gene expression on a global scale could vary in several cell types under diverse physiological and developmental conditions.

Global or specific DNA methylation correlated with HCy levels may contribute to vascular damage by different mechanisms. First, DNA methylation is recently emerging as a primary regulator of inflammation and may reflect altered immune or inflammatory responses during atherosclerosis in several cell types. Leukocyte functions related to cardiovascular risk, including the expression of soluble mediators and surface molecules that direct the adhesion and migration of blood leukocytes in vascular tissues, has been shown to be controlled by aberrant global DNA methylation (Kim et al. 2010). Hcy activates vascular smooth muscle cell alterations by upregulating the secretion of mitogen platelet-derived growth factors through DNA demethylation in endothelial cells (Zhang et al. 2012).

Changes in DNA methylation have also been suggested as a potential mechanism for altered apoprotein A-I and apoprotein A-IV gene expression in mice with HHcy (Mikael et al. 2006). Finally, Hcy plays a potential role in increasing the methylation of the ATP-binding cassette transporter A1 through DNA MTs, whereas the methylation of Acyl-coenzyme A cholesterol acyltransferase-1 was decreased, producing an accumulation of cholesterol in foam cells (Liang et al. 2013).

Histone Modification and Chromatin Remodeling

Histones are basic proteins that facilitate the packaging of DNA into nucleosomes in the nucleus. Nucleosomes are the basic units of chromatin and are composed of DNA wrapped around a protein octamer containing two molecules of each canonical histone (H2A, H2B, H3, and H4). Different post-translational histone modifications may alter chromatin structure in the nucleosomes, affecting the transcription of the associated genes by altering transcription factor accessibility (Arrowsmith et al. 2012). Therefore, histones control chromatin dynamics and regulate gene expression in a specific manner. Post-translational modifications of histones include acetylation, methylation, ubiquitination, and phosphorylation. The balance between histone acetylation and deacetylation is among the most studied histone-related epigenetic mechanisms, and its interplay regulates important gene expression linked to cardiovascular disease development. Histone deacetylation plays an important role by mediating diverse functions of cell energy metabolism and genomic stability. Therefore, the expression of oxidized low-density lipoprotein receptors (Dje N'Guessan et al. 2009) and eNOS mRNA (Kheirandish-Gozal et al. 2013) and modulation of cholesterol biosynthesis and transport pathways are regulated by

chromatin-modifying enzymes (Shafaati et al. 2009). Histone methylation is influenced by histone MTs that transfer a methyl group from the cofactor SAM to lysine or arginine residues on histone tails. Although many studies have demonstrated that histone modifications play a role in atherosclerosis, limited evidence is available about the implication of HHcy in this setting. Interestingly, the restricted expression of eNOS to the vascular endothelium is determined in part by DNA methylation and histone modification (Matouk and Marsden 2008).

Homocysteine as an Epigenetic Regulator of microRNAs

MicroRNAs comprise a novel class of endogenous, small, noncoding RNA molecules of ~22 nucleotides, which negatively modulate gene expression. These noncoding RNAs have been shown to have diverse functions in DNA methylation, transcriptional regulation, alternative splicing, post-transcriptional modification, chromatin structure modification, and RNA-translation mechanisms. MicroRNAs regulate approximately 30 % of the genes in the human genome and are involved in cell differentiation, growth, proliferation, and apoptosis. They are key modulators of both cardiovascular development and angiogenesis. Consequently, the dysregulation of microRNA function may lead to cardiovascular diseases. Recent studies stress the potential role of microRNAs in vascular smooth muscle cell proliferation and apoptosis (Ji et al. 2007) and in the regulation of cholesterol homeostasis by modulating both HDL biogenesis in the liver and cellular cholesterol efflux (Rayner et al. 2010).

Dicer endonuclease (the terminal enzyme involved in the maturation of all microRNAs) expression is enhanced in HHcy, promoting a global increase in microRNA synthesis (Mishra et al. 2009). Thus, microRNA expression provides a plausible hypothesis to link Hcy with cardiovascular disease.

Conclusion

In summary, several hypotheses can explain HHcy-induced pathophysiology in various organs: (1) reduced oxidative defense and enhanced production of reactive oxygen species (ROS); (2) decreased NO bioavailability; and (3) alterations in gene expressions through epigenetic changes involving aberrant methylation. These three major mechanisms can produce (a) inflammation and its associated changes; (b) inhibition of NO and other key signaling pathways; and (c) enhanced ER stress. They can act at different levels in the vascular system (Fig. 4). It is possible that when Hcy accumulates abnormally, it may produce many of the changes listed above simultaneously. Nevertheless, the discussion persists about the chemical identity of the Hcy species directly implicated in tissue damage. At present, it's difficult to establish whether the toxic effects were derived from Hcy itself or from the abovementioned metabolic compounds promoted by Hcy increase (Fig. 3).

Potential Applications to Prognosis: Prognostic Value of Disturbed Homocysteine Metabolism as a Risk Factor for Vascular Diseases

Ideally, to declare Hcy as a biomarker, its determination should add value to existing tests and improve its ability to predict risks, enhancing the clinician's decision, and improve patient management. Although the study of Hcy is likely to be biologically informative about the mechanisms of vascular disease, its clinical value as a sensitive diagnostic and prognostic test remains uncertain. Over the past several decades, there has been remarkable consistency in observational studies showing that elevated Hcy is associated with increased risk of cardiovascular disease. Wald et al. (2002) found that for every 5 μmol/L increase in the serum Hcy concentration, the risk of ischemic heart disease increased 20–30 %. However, no risk reduction is found in Hcy-lowering trials and there is no evidence of any benefit in the use of folate or other B vitamins on cardiovascular outcomes.

A post hoc analysis of studies from the National Health and Nutrition Examination Survey and the Multi-Ethnic Study of Atherosclerosis trials showed that the predictive value of adding total Hcy levels to the Framingham risk score was associated with a better reclassification of approximately 20 % of patients at intermediate risk (Veeranna et al. 2011). Thus, this study could provide a sound rationale for using Hcy in cardiovascular risk assessment. However, a recent updated Cochrane database systematic review of 12 trials involving 47,429 low-risk patients receiving B-complex vitamins to lower Hcy levels did not significantly affect the risk of myocardial infarction, stroke, or death by any cause (Martí-Carvajal et al. 2013).

Most of these studies did not select patients based on a significantly elevated plasma Hcy level, an approach that may have influenced the results. In addition to the fact that these clinical trials may have been shorter in duration than appropriate for modulating chronic disease states, it is likely that reduction of the blood Hcy levels may be an oversimplified approach to a complex biological perturbation. Hcy concentrations are tightly controlled by the transmethylation cycle and its interaction with transsulfuration. Transmethylation reaction generates a methyl group that is available for DNA methylation, protein, lipid, and carbohydrate synthesis. Effects of folate and other B vitamins on DNA methylation are complex and influenced by the duration of treatment, dosage, the tissue involved, and baseline demographic features, such as age. Furthermore, effects on specific genes may be rather different. Redox potential is linked (Joseph and Loscalzo 2013) via the methionine-Hcy cycle and allied to folate/one-carbon metabolism. Due to this complexity, instead of focusing the efforts on lowering the Hcy level, further research should concentrate on regulating the disturbance of the redox-methylation balance seen in hyperhomocysteinemic states.

Emerging Role of Disturbed Homocysteine Metabolism as a Risk Factor for Other Diseases

Given the central role of Hcy in cellular function via oxidative stress and metabolism of hydrogen sulfide, donations of one-carbon units, and methylation reactions, it is easy to understand that impaired Hcy and folate metabolism are involved in the pathogenesis of a wide range of common diseases (Table 2). These include not only cardiovascular disease, cerebrovascular (stroke) disease (Holmes et al. 2011), and neural tube defects (Candito et al. 2008) but also other congenital birth defects, such as congenital heart disease, cleft lip and palate, spontaneous abortion, late pregnancy complications (abruptio placentae), different types of neurodegenerative (Ansari et al. 2014) and psychiatric diseases, osteoporosis, risk fractures, and cancer (Wu et al. 2014). A main focus of future research is to determine whether folic acid or other types of Hcy-lowering therapy will reduce the risk of these diseases.

Summary Points

- The only known physiological function for homocysteine is to serve as an obligatory sulfur-containing intermediary metabolite in the conversion of methionine into cysteine. The levels of homocysteine reflect the balance of a complex network of reactions that mediate methyl and sulfhydryl group exchange and that are very sensitive to B-group vitamin supply.
- The increase in circulating homocysteine, even in the low-moderate range, has widespread detrimental effects on the vascular system. However, the molecular basis of homocysteine toxicity is under discussion. Several potential but no exclusive mechanisms have been proposed for homocysteine itself and some related metabolites. At present, no unifying hypothesis for the atherogenic and thrombogenic effects of homocysteine is available.
- The level of circulating homocysteine is considered a major independent risk factor for vascular disease, but its significance as a surrogate marker for endothelial dysfunction or as a causative agent remains controversial.
- The main causes of excess homocysteine in plasma are the inadequate intake of B-group vitamins (folic acid, B12, B6, and riboflavin), renal dysfunction, and genetic allelic variants of the enzymes implicated in homocysteine metabolism.
- Folic acid and B vitamin supplementation are proven to be effective in lowering plasma homocyateine levels. However, at this point, a decisive relationship between these homocysteine-lowering therapies and the reduction of cardiovascular risk has not been sustained by extensive clinical trials.
- The number of disease processes in which abnormal homocysteine metabolism is observed is increasing progressively. Elevations of circulating homocysteine appear to be a common trait to many pathologies, most of them associated with oxidative stress.
- Future studies are needed to clarify the pathological significance of increased homocysteine levels. Whether excess homocysteine is a decisive pathogenic

factor or a biomarker of metabolic disturbances remains a promising area for investigation.

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Neutrophil Gelatinase Associated Lipocalin (NGAL) as a Biomarker for Cardiovascular Disease

18

Kevin Damman and Mattia A. E. Valente

Contents

Abstract

Neutrophil gelatinase-associated lipocalin (NGAL) is a member of the lipocalin family and is involved in protection against bacterial infections. High NGAL levels are also abundant during acute kidney injury, in cardiovascular disease and chronic inflammatory states. Elevated NGAL levels in urine and plasma are relatively strong predictors of subsequent acute kidney injury in patients with nephrological disease, coronary artery disease, and, to a lesser extent, heart failure. Furthermore, higher NGAL levels relate to poor outcome in a variety of patients populations. However, the potential clinical utility of plasma or urine NGAL levels remains unclear and is the focus of ongoing and future research.

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_15

Keywords

Neutrophil gelatinase-associated lipocalin • NGAL • Acute kidney injury • Tubular injury • Prognosis • Worsening renal function

Abbreviations

AKI	Acute kidney injury
AUC	Area under the curve
BNP	Brain natriuretic peptide
CIN	Contrast-induced nephropathy
CKD	Chronic kidney disease
COPD	Chronic obstructive pulmonary disease
CV	Cardiovascular
ESRD	End-stage renal disease
GFR	Glomerular filtration rate
HF	Heart failure
HR	Hazard ratio
IL	Interleukin
KIM-1	Kidney injury molecule 1
L-FABP	Liver-type fatty acid-binding protein
LVAD	Left ventricular assist device
MMP-9	Matrix metalloproteinase 9
NAG	*N*-acetyl-beta-D-glucosaminidase
NGAL	Neutrophil gelatinase-associated lipocalin
TIMP	Tissue inhibitor metalloproteinase
WRF	Worsening renal function

Key Facts About Acute Kidney Injury

- Acute kidney injury or AKI is defined by increases in serum creatinine and/or decrease in urine output.
- Although different criteria exist, the most commonly used definition is a creatinine increase 1.5–1.9 times baseline over 1–7 days or $\geq$26.5 μmol/L (0.3 mg/dL) increase within 48 h.
- Acute kidney injury has been associated with poor outcome.
- Early identification of patients who are at risk of developing AKI remains difficult.
- NGAL is thought to be a strong predictor of AKI but only in specific conditions.

Key Facts About Heart Failure

- Heart failure is not a disease; it is a complex of symptoms caused by a multitude of diseases.

- The hallmark of heart failure is a myriad of symptoms of congestion, including dyspnea, edema, and fatigue.
- There are different types of heart failure: heart failure with preserved ejection fraction and heart failure with reduced ejection fraction.
- There is no effective evidence-based therapy for heart failure with preserved ejection fraction.
- Prognosis of patients with heart failure has improved greatly in the past decades, but around 20–30 % of patients still die within 5 years of the diagnosis.
- Worsening renal function occurs in 10–15 % of patients with heart failure and has been associated with poor outcome.

Definitions

Acute kidney injury (AKI) Situation of rapid decline in renal function, mostly defined using increases in serum creatinine or decreases in urine output over a short period of time

Contrast-induced nephropathy Specific condition of acute kidney injury that occurs 24–72 h after exposure to iodinated contrast medium

Heart failure Clinical syndrome where structural and/or functional heart disease results in underperfusion of vital organs with subsequent salt and water retention, leading to symptoms of congestion

Worsening renal function Deterioration in renal function in heart failure, less severe compared with acute kidney injury and mostly over longer period of time

Introduction

Neutrophil gelatinase-associated lipocalin (NGAL) is a molecule in the lipocalin family, a group of proteins with the ability to bind specific small lipophilic ligands. Thus, lipocalins play a role in protein transport and serve in different biological functions such as vitamin delivery, pheromone transport, and prostaglandin synthesis (Flower 1994; Kjeldsen et al. 2000; Schmidt-Ott et al. 2007). NGAL is a 21 kDa protein and exists in mono-, homo-, or heterodimers. In humans, NGAL is secreted from granules in body cells, especially neutrophils, hence the name neutrophil-GAL. NGAL is expressed by multiple organs and tissue types, but primary (low grade) NGAL expression has been observed in the trachea, stomach, lungs, spleen, and bone marrow (Schmidt-Ott et al. 2007). To a lesser extent, NGAL expression has been seen in thee proximal renal tubules (Cowland and Borregaard 1997). Elevated NGAL levels have been observed in bacterial overgrowth or bacterial infection. In fact, the study that discovered NGAL found that it played an important role in the protection against certain

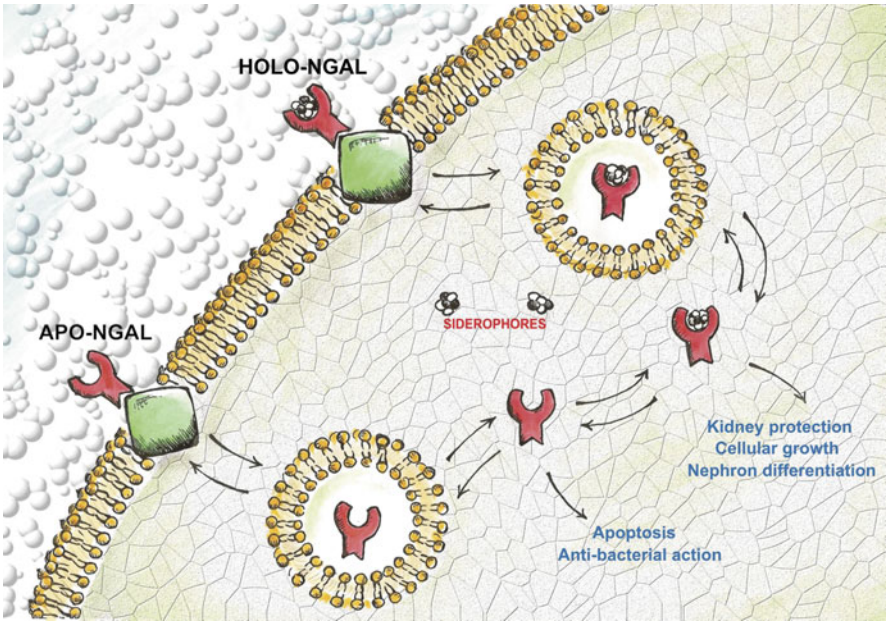

Fig. 1 NGAL cellular turnover and iron traffic through siderophores. NGAL interacts with specific receptors (24p3R or megalin) as a complex with iron siderophores (Holo-NGAL) or alone (Apo-NGAL). After internalization, Holo-NGAL is able to release the iron it carried into the cytoplasm. Apo-NGAL can capture intracellular iron siderophores and transport these to the extracellular space, thus depriving the cell of its iron reserves (Reprinted from Bolignano et al. (2008), Copyright (2008), with permission from Elsevier)

bacteria (Goetz et al. 2002). NGAL also plays a key role in iron transport through formation of a complex with siderophores – an iron-containing/iron-scavenging protein (Fig. 1) (Bolignano et al. 2008). This mechanism removes iron from certain specific bacteria that require iron for growth, differentiation, and proliferation, thus potentially influencing the course of bacterial infection. NGAL knockout mice were found to be at increased risk of bacterial infections, confirming this mechanism (Goetz et al. 2002; Flo et al. 2004). Increased plasma NGAL levels have since been observed in other disease states with low-grade inflammation, such as chronic obstructive pulmonary disease (COPD), inflammatory bowel disease, cancer, SLE, myocardial infarction and coronary artery disease, heart failure, and, most importantly, acute kidney injury (AKI). In AKI, NGAL levels have been found to be increased 1000-fold compared with baseline levels (Schmidt-Ott et al. 2007). Besides iron scavenging, the specific functions of NGAL remain incompletely understood.

In the present chapter, we will review available data on the utility of NGAL as diagnostic, prognostic, and therapeutic marker of cardiovascular disease.

NGAL in Kidney Disease

The majority of research on NGAL in cardiovascular disease has been conducted in patients with either chronic or acute kidney disease. In tubular injury, NGAL was found to be among the most upregulated genes, a signal that was confirmed at the mRNA level (Mishra et al. 2003). NGAL levels were shown to be extremely elevated in ischemic tubular injury in both plasma and urine. In their pivotal 2003 study, Mishra and colleagues also characterized NGAL response to renal ischemia-reperfusion injury, observing a tenfold increase in rat kidney NGAL mRNA after ischemia, along with a parallel 4–12-fold increase in renal NGAL protein content (unilateral vs. bilateral ischemia). Importantly, in this early study, NGAL was also already present in urine of mice and rats within 2 h of ischemia-reperfusion injury and preceded any changes in known markers of tubular damage. Even mild ischemia was associated with increases in urinary NGAL concentrations. Early signals of increased urinary levels of NGAL were also observed in humans with acute renal injury. The same group published a landmark paper on NGAL and its potential utility in clinical practice 2 years later (Mishra et al. 2005). In this study, which did not encompass patients with renal disease but included children undergoing cardiopulmonary surgery, NGAL was evaluated as an early marker of AKI. The phenomenon of AKI is frequently observed in critically ill patients, patients who undergo cardiac surgery, following iodinated contrast during procedures (contrast-induced nephropathy), and as solitary phenomenon. It is associated with strongly increased adverse event rates, and to date, the (early) identification of patients at risk of AKI has proven challenging. In this particular study, 28 % of patients experienced AKI based on serum creatinine. Plasma and also urine NGAL levels were significantly increased in patients who developed AKI. This increase was observed as early as 2 h after the end of surgery and was also quite significant (20 (plasma) to 100 (urine) times baseline levels) (Fig. 2). This resulted in extremely favorable diagnostic characteristics for NGAL, with an area under the curve (AUC) for the diagnosis of AKI of 0.998. Such incredible results have not since been replicated for NGAL and are probably too good to be true; however, they do indicate the possible strength of NGAL as marker of AKI. A meta-analysis of a large number of studies has shown reasonable predictive ability for NGAL in the diagnosis of AKI (Fig. 3) (Haase et al. 2009).

In a different study in a pediatric population, this time in patients with stage 2–4 chronic kidney disease (CKD), NGAL was evaluated as biomarker for CKD severity (Mitsnefes et al. 2007). In this small study of 45 patients, serum NGAL levels were strongly correlated with both cystatin C and measured and estimated glomerular filtration rate (GFR). NGAL was able to identify patients with lower GFR with good accuracy (AUC = 0.88 for eGFR < 30 mL/min/1.73 m^2 and AUC = 0.86 for eGFR < 60 mL/min/1.73 m^2).

More evidence from NGAL as marker of tubulointerstitial damage is found in chronic kidney disease. In a small study of 70 IgA nephropathy patients and 40 controls, Ding and colleagues evaluated whether NGAL could distinguish

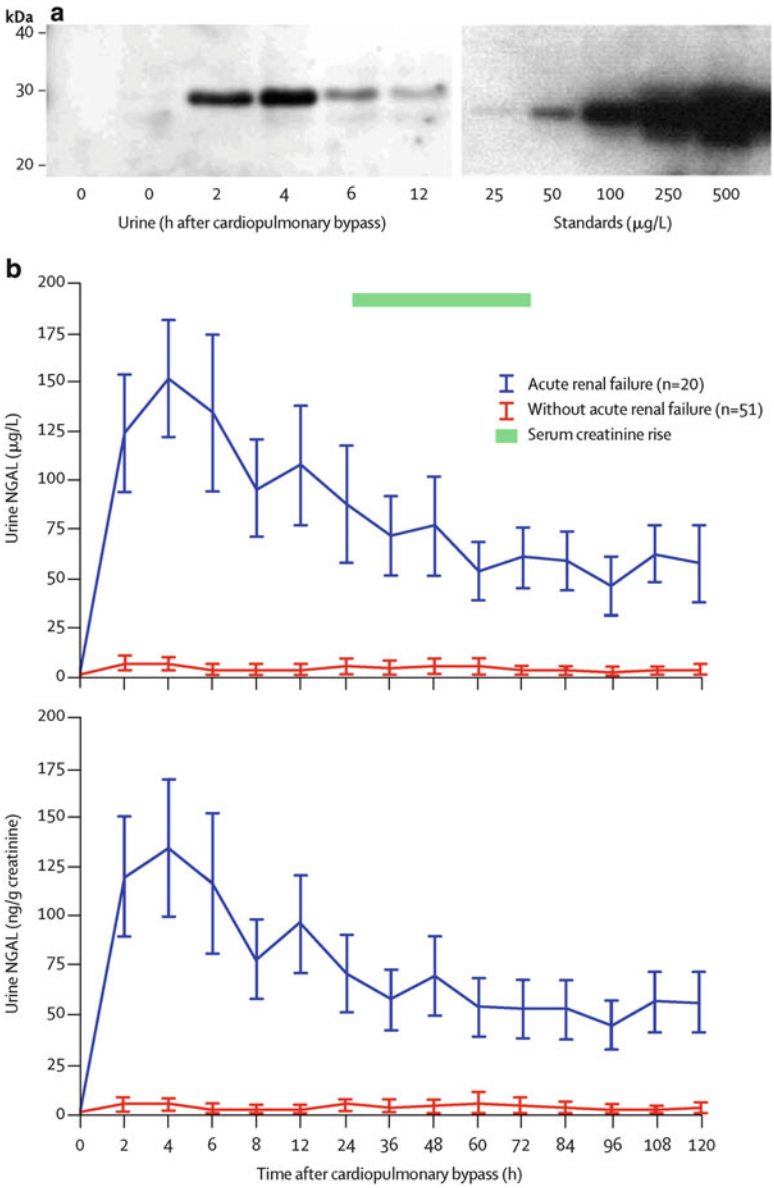

Fig. 2 Urine NGAL and acute kidney injury in children undergoing cardiopulmonary bypass grafting. (**a**) Representative western blot of urine samples obtained at various time points after cardiopulmonary bypass from a patient who subsequently developed acute renal failure. Both blots were probed with a monoclonal antibody to human NGAL. (**b**) Mean urine NGAL concentrations at various time points after cardiopulmonary bypass (*upper*) and corrected for urine creatinine excretion (*lower*). Error bars are SE. Data from 71 children undergoing cardiopulmonary bypass grafting (Reprinted from Mishra et al. (2005), Copyright (2005), with permission from Elsevier)

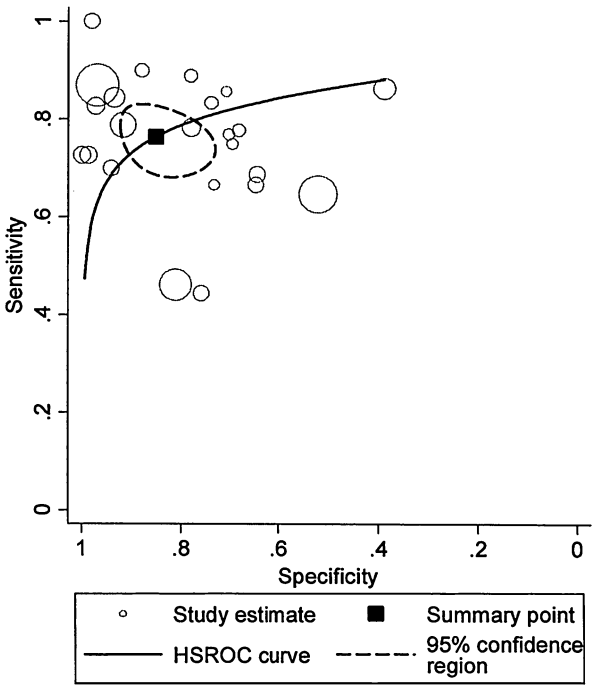

Fig. 3 Pooled performance of NGAL to predict acute kidney injury. Hierarchical summary receiver operating characteristic (HSROC) plot of neutrophil gelatinase-associated lipocalin (NGAL) to predict acute kidney injury across settings. Based on combined sensitivity (95 % confidence interval [CI]) and specificity (95 % CI) weighted for sample size of each data set reflected by the size of the *circles*, showing average sensitivity and specificity estimate of the study results (*solid square*) and a 95 % confidence region around it. Reflects meta-analysis on different studies and data sets (Reprinted from Haase et al. (2009), Copyright (2009), with permission from Elsevier)

between different grades of IgA nephropathy (Ding et al. 2007). The study evaluated urine, serum, and tissue NGAL levels in these patients. Both urinary and serum NGAL levels showed a stepwise increase with greater disease severity. This observation was further strengthened by findings that tissue NGAL levels were also elevated in these patients and that the amount of tissue NGAL correlated strongly with urinary NGAL levels.

Similar observations were made in patients with different etiology of CKD: autosomal-dominant polycystic kidney disease (Bolignano et al. 2007). Both urinary and serum NGAL levels were increased above control levels, and patients with more severe disease showed even higher NGAL levels.

Importantly, NGAL has not only been analyzed in a cross-sectional setting but also as marker of disease progression and clinical outcome. In two analyses from the Chronic Renal Insufficiency Cohort (CRIC) Study, urinary NGAL was evaluated in 3386 patients with estimated GFR between 20 and 70 mL/min/1.73 m^2 (Liu et al. 2013, 2015). First, the authors evaluated whether urinary NGAL levels could predict progression of renal disease defined as halving of eGFR or incident end-stage renal disease (ESRD). Urinary NGAL was found to improve risk stratification of patients at risk of progressive renal disease by 24 % using net reclassification index, but overall C-statistics did not improve. Patients with urinary NGAL levels >49.5 ng/mL had a 70 % increased risk of progression of renal disease, independently of other clinical risk factors, eGFR and albuminuria. Overall, the authors

conclude that NGAL does identify high-risk individuals but that the total additional information gained is probably limited. The second analysis from CRIC evaluated whether urinary NGAL levels can predict cardiovascular events other than progression of CKD (Liu et al. 2015). For this analysis, the primary outcome was the first occurrence of either heart failure (HF), atherosclerotic events (myocardial infarction, ischemic stroke, peripheral artery disease), or death. In unadjusted analysis, patients with the highest quintiles of NGAL (>49.5 ng/mL) had a significantly increased risk of all individual events, with hazard ratios ranging between 2.71 (HF), 2.43 (atherosclerotic event), and 1.96 (death). However, after adjustment for confounders, including eGFR and albuminuria, urinary NGAL only remained significantly and independently associated with incident atherosclerotic events (HR 1.83, 95 % confidence interval 1.20–2.81).

Some of these results have been replicated in a different study in 473 Taiwanese CKD patients (Lin et al. 2015). Over a mean period of a little more than 3.5 years, 26 % of patients progressed to end-stage renal disease. Urinary NGAL levels were strong predictors of progression to ESRD, with the highest NGAL tertile showing a 300 % increase in the risk of ESRD. Similarly, urinary NGAL levels were significant predictors of cardiovascular events (acute coronary syndrome, stroke, peripheral artery disease, HF, or CV death) but not independently of other clinical risk factors.

The question arises why NGAL levels increase during chronic kidney disease, but more markedly in acute kidney injury and more specifically during ischemia. As we have already mentioned, NGAL plays a role in iron transport by binding siderophores, and a study by Mori and colleagues suggest that at least part of this function may be also the reason for the strong increases in NGAL levels in acute kidney injury (Mori et al. 2005). In their extensive experiments, the authors showed that the presence of the NGAL-siderophore complex may attenuate ischemia-reperfusion injury. They also showed that NGAL activates different protective pathways and suppresses more damaging pathways, resulting in rescue from cell damage and death. This suggests that the expression of NGAL in the event of acute injury could actually be a form of protection. Exactly how NGAL may prevent ischemic cell death and apoptosis is unknown.

NGAL in Heart Disease: Coronary Artery Disease

Kidney disease is prevalent in patients with heart disease, and therefore, NGAL has been evaluated in patients with coronary artery disease or undergoing cardiopulmonary bypass grafting. NGAL has also been evaluated during and after coronary angiography, where a minority of patients develops contrast-induced nephropathy (CIN), a form of acute kidney injury.

Bachorzewska and colleagues evaluated the time course of NGAL levels in serum and plasma in patients scheduled to undergo elective percutaneous intervention due to significant coronary stenoses (Bachorzewska-Gajewska et al. 2006). Serum NGAL levels increased early after PCI; after 2 and 4 h, serum NGAL levels were significantly elevated compared with baseline and dropped to baseline levels within

2 days. A similar pattern was observed with urine NGAL, although this effect was somewhat delayed (12 h). In a follow-up study by the same authors, NGAL was evaluated as possible predictor of CIN in 60 patients who underwent a percutaneous intervention (Bachorzewska-Gajewska et al. 2007). In agreement with their earlier findings, overall serum and urine NGAL levels increased in all patients. CIN developed in 10 % of patients, in whom NGAL levels rose significantly higher compared with controls in both serum (2 h) and urine (4 h). This resulted in 90 % sensitivity and 74 % specificity to detect CIN using serial NGAL measurements. More recent data come from a study by Torregrosa et al., who evaluated multiple biomarkers for AKI assessment in acute coronary syndrome patients undergoing coronary angiography vs. cardiac surgery patients (Torregrosa et al. 2015). The authors evaluated NGAL, kidney injury molecule 1 (KIM-1), and liver-type fatty acid-binding protein (L-FABP) in urine 12 h after intervention in 193 patients. In their analyses, NGAL levels were elevated fivefold in patients who developed AKI compared with those who did not, while KIM-1 and L-FABP levels increased only slightly. This resulted in an area under the curve of 0.958 and 0.916 for the diagnosis of AKI with NGAL in patients who underwent coronary angiography and cardiac surgery, respectively. These data on AKI in cardiac surgery have been confirmed in different studies. NGAL and interleukin-18 levels were found to increase significantly in cardiac surgery patients who developed AKI (Parikh et al. 2006). In this case, urine NGAL was measured and increased almost 100-fold in patients who developed AKI. IL-18 levels increased in a similar fashion, but the increase in urinary NGAL levels was observed as early as 2 h after the index event. Together, NGAL and IL-18 provided additive and independent predictive information for AKI. Further confirmation comes from data on 82 patients who underwent cardiac surgery (Wagener et al. 2006). While urinary NGAL levels were low in all patients, patients who developed AKI had significantly increased levels as early as 1 h after surgery. This preceded the increase in serum creatinine by over a day (serum creatinine rose significantly at 3 days), suggesting NGAL may serve as an early marker for AKI in these patients. This was confirmed by the area under the curve (AUC) for AKI, with a value of 0.8 for NGAL measured 18 h after surgery, suggesting acceptable sensitivity and specificity for AKI. In this analysis, the negative predictive value was particularly high, as patients without AKI also showed a slight rise in urinary NGAL, which was gone by 18 h.

Overall, these (small) studies seem to indicate that NGAL (either urine or serum) measured early after surgery or coronary angiography is able to identify patients at risk for CIN/AKI. They do not, however, indicate whether specific therapy initiated early after diagnosis of CIN/AKI using NGAL levels can improve outcome and possibly reduce the incidence of CIN/AKI. Further research on this particular subject is still needed.

The predictive value of NGAL levels has not only been studied in patients with coronary artery disease. Some researchers have also evaluated the value of NGAL in atherosclerosis. Since NGAL modulates the activity of matrix metalloproteinase 9 (MMP-9), which is involved in the stability of atherosclerotic plaques, Hemdahl and colleagues evaluated the NGAL/MMP-9 complex in mice that developed

myocardial infarction due to hypoxic stress (Hemdahl et al. 2006). They found that NGAL RNA and gene expression were significantly elevated in these mice, suggesting NGAL may play a role in destabilizing plaques by augmenting MMP-9 protease activity, which is found in unstable plaques. The authors confirmed part of this hypothesis, demonstrating the colocalization of NGAL and MMP-9 within macrophages in the lipid core of human atherosclerotic plaques. Another small study showed that the MMP-9/NGAL complex is also present in cardiomyocytes but that the concentration of the complexes is similar in heart failure compared with controls (Kiczak et al. 2014).

Overall, there are some indications that intracellular NGAL levels, together with MMP-9 and TIMP, may play a role in the stability of atherosclerotic plaques and could therefore be important in the pathophysiology of coronary artery disease. However, the limited, preclinical data available to date still require validation and further exploration.

NGAL in Heart Disease: Heart Failure

The prediction of significant deterioration in renal function is particularly important heart failure (HF). In HF, these increases in serum creatinine are termed worsening renal function (WRF), as the increases in serum creatinine and changes in urine output are markedly different (mostly smaller) compared with AKI (Damman et al. 2014a). The heterogeneity of HF populations and the effects of multiple HF therapies on the kidney have contributed to the challenge of predicting meaningful WRF. Despite the often smaller changes, patients who develop clinically significant WRF with a parallel deterioration in clinical status have strongly increased mortality rates (Damman et al. 2014b). Additionally, both reduced glomerular function and albuminuria are frequently present in patients with HF and have been associated with poor clinical outcome (Jackson et al. 2009). Therefore, the first studies in HF evaluated whether these patients also experience tubular damage/dysfunction, which is thought to arise when renal blood flow is compromised in HF. In a small cohort of patients with HF with reduced ejection fraction, urinary NGAL levels were significantly elevated compared with age- and sex-matched controls and were related to the severity of HF (Damman et al. 2008). Urinary NGAL levels were also elevated in a different cohort of patients with ischemic HF, where higher levels were found in patients with more severe HF (Poniatowski et al. 2009). In the largest cohort of patients with HF with available urine samples, tubular damage was prevalent, as indicated by elevated KIM-1, N-acetyl-beta-D-glucosaminidase (NAG), and NGAL levels (Damman et al. 2011a). Additionally, all tubular markers predicted clinical outcome in this cohort of HF patients, including NGAL, although urinary NAG was the strongest predictor among the three markers. Other data in chronic HF comes from the Cleveland Clinics, where serum NGAL levels were evaluated in 130 patients (Shrestha et al. 2011). NGAL levels were elevated compared with controls and predominantly associated with lower estimated GFR. In addition, serum NGAL was associated with clinical outcome, but not after adjustment for

GFR. In a different manuscript evaluating the same population, the authors also showed that NGAL levels associate with markers of anemia (Shrestha et al. 2012a). The dependency of the prognostic information of serum NGAL on renal function was further supported by data from the CORONA study, where NGAL levels were evaluated in 1415 ischemic HF patients (Nymo et al. 2012). Higher NGAL levels were associated with more severe HF and more severe renal dysfunction. In univariate analysis, NGAL was associated with the composite clinical endpoint but not after multivariate adjustment for GFR. Serum NGAL levels are specifically increased in patients with severely depressed left ventricular function, as evaluated in patients who receive left ventricular assist devices (LVAD) (Pronschinske et al. 2014). Interestingly, NGAL levels decrease with LVAD therapy, probably due to improved renal perfusion. On the other hand, both urinary and serum NGAL levels were not affected by changes in diuretic therapy (withdrawal and re-initiation), while urinary NAG and KIM-1 levels were (Damman et al. 2011b). Urinary NGAL levels were significant predictors of the occurrence of WRF in patients with chronic HF, but this relationship did not persist after adjustment for confounders (Fig. 4) (Damman et al. 2013). On the other hand, urinary KIM-1 levels were the most important predictors of WRF in these patients, outperforming even eGFR. This suggests urinary NGAL levels in chronic HF might provide a different signal compared with urinary KIM-1 (and NAG for that matter). Finally, in experimental HF, NGAL levels were found to be elevated in cardiomyocytes, a finding

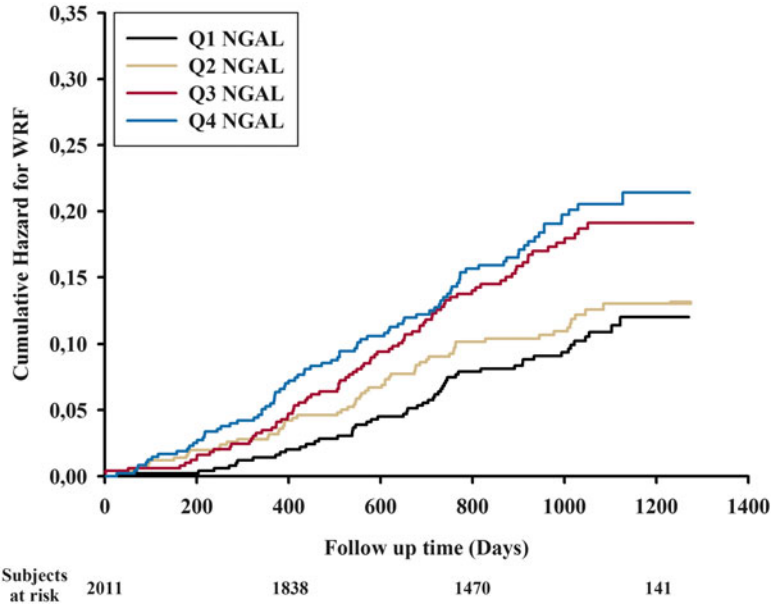

Fig. 4 Association between urine NGAL and occurrence of worsening renal function in chronic heart failure. *Q* quartile, *WRF* worsening renal function. Shows univariate association (Reprinted from Damman et al. (2013), Copyright (2013), with permission from Elsevier)

confirmed in patients with clinical chronic HF, suggesting that not only circulating NGAL levels but also intracardiac expression of NGAL play some role in the pathophysiology of HF (Yndestad et al. 2009).

In acute HF, most if not all studies evaluated plasma rather than urine NGAL levels. Pallazuolli and colleagues evaluated admission NGAL levels and the ability to predict WRF in patients with acute HF (Palazzuoli et al. 2014). The authors found elevated admission NGAL levels in 179 patients. More importantly, NGAL levels were strongly elevated in patients who subsequently developed WRF, resulting in a 92 % sensitivity for WRF with a cutoff for NGAL of 134 ng/mL. At almost the same cutoff level (130 ng/mL), NGAL levels predicted poor clinical outcome in these patients. Aghel and colleagues found almost the same cutoff being predictive for WRF (at 140 ng/mL, odds ratio 1.92, 95 % confidence interval 1.23–3.12, $P = 0.004$), while Alvelos et al. showed that an NGAL level 167.5 ng/mL was associated with increased mortality (Aghel et al. 2010; Alvelos et al. 2013). Another study evaluated both urine and serum NGAL levels and showed that both predicted WRF in acute HF (Shrestha et al. 2012b). On the other hand, two studies showed no association between baseline NGAL and either WRF or clinical outcome (Breidthardt et al. 2012; Maisel et al. 2011) (Fig. 5). However, discharge plasma NGAL levels were associated with clinical outcome, even independently of brain natriuretic peptide (BNP) levels. The latter relationship between discharge serum NGAL and subsequent outcome was further evaluated in 562 patients who were hospitalized with HF (van Deursen et al. 2014). That analysis indicated that serum NGAL levels were predictive of long-term all-cause mortality, even in patients with normal GFR (Fig. 6).

Finally, one small study evaluated NGAL as a marker of a totally different pathway. Serum NGAL levels might play a role in depression, and therefore, this small study in around 100 chronic HF patients showed that serum NGAL levels were

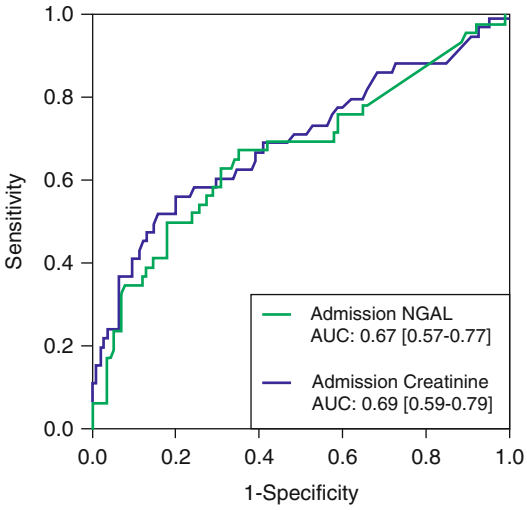

Fig. 5 Predictive ability of NGAL for acute kidney injury in acute heart failure. Represents data on 207 patients with acute heart failure, where 29 % experienced acute kidney injury (Reprinted under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0). Original published by Breidthardt et al. (2012))

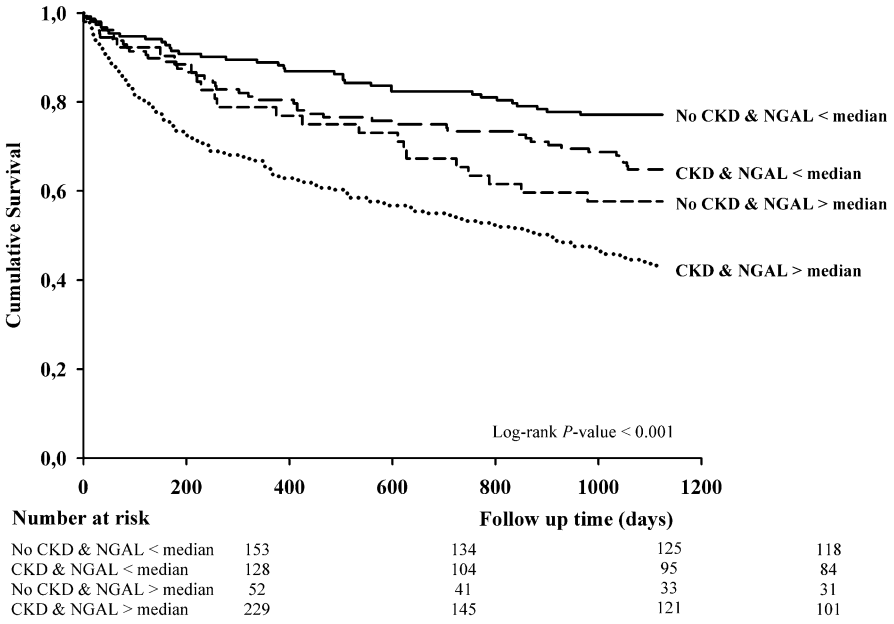

Fig. 6 NGAL and mortality in heart failure. *CKD* chronic kidney disease, *NGAL* neutrophil gelatinase-associated lipocalin. Kaplan-Meier curves showing the association between all-cause mortality and low/high estimated glomerular filtration rate (cutoff point, 60 mL/min per 1.73 m^2) and low/high NGAL (cutoff point, 84.62 ng/mL). Hazard ratio vs. no CKD/NGAL<median for CKD/NGAL<median, 1.65, 95 % confidence interval (CI), 1.01–2.56, $P = 0.027$; for no CKD/NGAL median, 2.05, 95 % CI, 1.20–3.49, $P = 0.009$; and for CKD/NGAL median, 3.26, 95 % CI, 2.24–4.74, $P < 0.001$. Data on 562 patients with heart failure (Adapted with permission from Lippincott Williams and Wilkins/Wolters Kluwer Health: van Deursen et al. (2014))

associated with more severe depressive symptoms, as well as more severe HF, although the magnitude of the association was small (Naude et al. 2014).

In summary, urinary and serum NGAL levels are (a) increased in patients with acute and chronic HF; (b) seem to be important and early predictors of WRF in acute but not chronic HF; (c) relate to all-cause mortality, mostly in the chronic outpatient setting; and (d) are an expression of NGAL in cardiomyocytes that is increased in HF. The potential clinical usefulness of NGAL determination in HF patients is still unclear, and results from the observational AKINESIS study are eagerly awaited.

Potential Applications to Other Diseases or Conditions

NGAL levels have also been evaluated in patients with atherosclerotic cerebrovascular disease. Elevated plasma NGAL levels have been observed in patients with cerebrovascular accidents or transient ischemic attacks and are associated with poor

clinical outcome over time (Anwaar et al. 1998a, b; Cruz et al. 2012). These levels were also shown to remain stable over time.

In the emergency department, NGAL has been used to try to identify patients at risk for AKI. Devarajan et al. evaluated 616 patients, of whom 21 % developed AKI. NGAL plasma levels were strong and independent predictors of AKI, especially for severe AKI (Soto et al. 2013). Patients with strongly increased NGAL levels (133 ng/mL) showed an almost tenfold increase in the incidence of AKI. In the intensive care unit, plasma and urine NGAL levels were shown to be predictive of AKI, and their diagnostic value was better at 24 h compared to baseline.

Summary

NGAL has been studied extensively during the past decade. It has been implicated in inflammatory disorders, bacterial infection, and, most importantly, as a marker and predictor of AKI and outcome in cardiovascular disease, including kidney disease, coronary artery disease, and HF. In general, plasma and urine NGAL levels have some promising characteristics and have been shown to predict the occurrence of AKI and WRF in HF, as well as clinical outcome in different diseases. However, not all studies have shown consistent results, and no studies have evaluated NGAL level-driven treatment decisions. Therefore, the potential clinical utility of plasma or urine NGAL levels remains and is the focus of ongoing and future research.

Summary Points

- NGAL is a member of the lipocalin family and has bacteriostatic properties.
- NGAL is primarily produced in the proximal tubule during situations of acute (ischemic) kidney injury but is present in a variety of tissues.
- Elevated plasma or urine NGAL has been shown to be a good predictor of subsequent acute kidney injury in different patient populations.
- Higher NGAL levels relate to poor outcome in patients with (chronic) heart failure.
- Administration of NGAL ameliorates renal ischemia-reperfusion injury.
- The clinical utility of plasma or urine NGAL is still under debate.

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Plasma Testosterone and Dihydrotestosterone as Markers of Heart Disease and Mortality in Older Men

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_38

Abstract

Testosterone (T) is the primary male sex hormone, exerting its effects directly or following conversion to dihydrotestosterone (DHT) which is a more potent ligand for the androgen receptor. Circulating concentrations of T are lower in older compared to younger men, and lower T is associated with a range of poorer health outcomes. Several studies have identified lower circulating T as a predictor of cardiovascular disease (CVD) risk. Concentrations of DHT are preserved in older men, but associations of DHT with health outcomes in this expanding demographic group are relatively unexplored. Recent studies have utilized mass spectrometry to accurately measure circulating T and DHT in large population-based cohorts of older men. Lower T has been associated with CVD events and all-cause mortality. Lower DHT concentrations are associated with higher mortality from ischemic heart disease. However, interventional studies of T supplementation have not been powered for the outcome of CVD events or mortality. Therefore, while reduced T and DHT are robust biomarkers for heart disease and related mortality, additional studies are required to determine causality and assess the role of T therapy in older men without proven androgen deficiency.

Keywords

Testosterone • Dihydrotestosterone • Estradiol • Cardiovascular disease • Ischemic heart disease • Myocardial infarction • Stroke • Mortality

Abbreviations

ARIC study	Atherosclerosis Risk in Communities study
BMI	Body mass index
CHD	Coronary heart disease
CHS	Cardiovascular Health Study
CI	Confidence interval
CVD	Cardiovascular disease
DHT	Dihydrotestosterone
E2	Estradiol
EMAS	European Male Aging Study
GC	Gas chromatography
HIMS	Health In Men Study
HR	Hazard ratio
IHD	Ischemic heart disease
LC	Liquid chromatography
LH	Luteinizing hormone
MrOS	Osteoporotic fractures in men
MS	Mass spectrometry
Q	Quartile

RCT	Randomized controlled trial
SHBG	Sex hormone-binding globulin
T	Testosterone
WHR	Waist/hip ratio

Key Facts Regarding Hormones and Cardiovascular Risk

- Testosterone (T) is the primary male sex hormone, exerting its effects directly or following conversion to dihydrotestosterone (DHT) which is a more potent ligand for the androgen receptor.
- Circulating concentrations of T are lower in older compared to younger men, and lower T is associated with a range of poorer health outcomes.
- Several studies have identified lower circulating T as a predictor of cardiovascular disease (CVD) risk.
- Recent studies have utilized mass spectrometry to accurately measure circulating T and DHT in large population-based cohorts of older men.
- Lower T and DHT concentrations are associated with higher incidence of stroke, while lower DHT concentrations are associated with increased mortality from ischemic heart disease.
- Interventional studies of T supplementation have not been powered for the outcome of CVD events or mortality, and meta-analyses of RCTs in general have not found T supplementation to be associated with excess cardiovascular adverse effects.
- While reduced T and DHT are robust biomarkers for heart disease and related mortality, additional studies are required to determine causality and assess the role of T therapy in older men without proven androgen deficiency.

Definitions

Adverse events These are incidents occurring following administration of a medication or during the course of a randomized controlled trial, which have a negative impact on health and well-being.

Androgen An androgen is a hormone which is responsible for male characteristics, such as pubertal development during childhood and adolescence and virilization and body composition in adulthood.

Cardiovascular disease This results from disease of the arteries and the heart and manifests with events such as heart attack or stroke. These events can result in hospitalization, ill-health, or death.

Longitudinal cohort study This is a study in which a large number of participants are recruited and then followed for a period of time. Hormones can be measured at the time of recruitment, and baseline hormone concentrations related to health outcomes occurring over time.

Mass spectrometry Gas chromatography-mass spectrometry and liquid chromatography-tandem mass spectrometry are accurate methods of measuring the concentration of androgens in the blood.

Randomized placebo-controlled trial This is a study where participants are allocated to active treatment or placebo in a random manner, such that any participant may receive one or either therapy. Trials are commonly conducted in a "double-blind" fashion in which neither the investigator treating the participant nor the participant is aware of which treatment is being given, in order to avoid influencing the result of the trial.

Testosterone therapy This is the administration of testosterone by application of transdermal gel or liquid or by intramuscular injections, to men who have diseases of the pituitary gland or the testes which result in their having low testosterone concentrations.

Introduction

Testosterone (T) is produced by the testis under the stimulation of pituitary luteinizing hormone (LH) and circulates largely bound to sex hormone-binding globulin (SHBG) and albumin, with a small fraction unbound or free (Bhasin 2008). T undergoes conversion by 5α-reductase to dihydrotestosterone (DHT), a more potent ligand for the androgen receptor, and by aromatase to estradiol (E2) a ligand for estrogen receptors (Lakshman et al. 2010). Therefore, biological actions of T flow from the function of the hypothalamic-pituitary-gonadal axis to the circulation of T and its active metabolites DHT and E2 and to tissue effects which regulate male sexual development, virilization, and body composition in adult men (Yeap et al. 2012b). Of note, circulating T in men declines with age (Harman et al. 2001; Feldman et al. 2002). There is ongoing debate over whether this is an effect of age per se or whether this change reflects the accumulation of obesity or age-related morbidities which are reflected in lower T concentrations (Sartorius et al. 2012; Shi et al. 2013). However, even healthy older men exhibit lower circulating T concentrations compared to reproductively normal younger men (Sikaris et al. 2005; Yeap et al. 2012a). In a study of 124 healthy, reproductively normal men aged 21–35 years, the reference interval for T assayed using mass

spectrometry was 10.4–30.1 nmol/L (Sikaris et al. 2005). By contrast, in a study of 394 men aged 70–89 years who reported excellent or very good health with no history of smoking, diabetes, cardiovascular disease (CVD), cancer, depression, or dementia, the reference interval for plasma T assayed using mass spectrometry was 6.4–25.6 nmol/L (Yeap et al. 2012a). Thus, advancing age is associated with both lower circulating T and increasing medical comorbidity (Yeap et al. 2012b), raising the question as to whether reduced exposure to T might contribute to declining health in aging men.

Measurement of Sex Hormones in Men

For the optimal evaluation of a biomarker, standardization of sample collection procedures and an accurate assay methodology are important. There is circadian variation in circulating T concentrations, which are higher in the morning and lower in the evening (Diver et al. 2003; Brambilla et al. 2009). The diurnal variation in DHT and E2 is much less compared to T in men in both middle-aged and older men (Brambilla et al. 2009). In an analysis of men undergoing glucose tolerance testing, T concentrations at 2 h post-challenge were lower than baseline leading those authors to recommend fasting blood tests for men being investigated for androgen deficiency (Caronia et al. 2013). With regard to assay methodology, automated immunoassays of T tend to exhibit nonspecificity and method-dependent bias (Wang et al. 2004; Sikaris et al. 2005). Therefore, mass spectrometry is the preferred assay methodology for sex steroids such as T (Handelsman and Wartofsky 2013). Similar considerations apply to preferring mass spectrometry over immunoassay for DHT and E2, as in men these metabolites of T circulate at much lower concentrations compared with T. Therefore, early morning collection of blood samples and use of mass spectrometry assays for sex steroids are most appropriate, as was performed in the studies establishing reference ranges for T in younger men (Sikaris et al. 2005) and T, DHT, and E2 in older men (Yeap et al. 2012a). There is ongoing discussion over the value of assessing free or unbound T both in the clinical setting and as a biomarker for epidemiological studies. SHBG increases with age and is lower in the setting of insulin resistance and obesity; thus, there are scenarios where consideration of free T may be informative (Cooper et al. 2015). However, measurement of circulating free T by equilibrium dialysis is labor-intensive and not routinely performed; instead, free T is commonly calculated using mass action or empirical equations (Vermeulen et al. 1999; Ly et al. 2010). Depending on the method of calculation, calculated free T can vary from measured free T which is a disadvantage (Ly et al. 2010). In contrast to declining circulating T, concentrations of DHT and E2 tend to be stable in middle-aged and older men (Feldman et al. 2002; Jasuja et al. 2013).

Androgens and Cardiovascular Events

Cohort Studies Using Immunoassays for Sex Steroids

Longitudinal cohort studies examining the association of sex hormones at baseline with the incidence of CVD events during follow-up are summarized in Tables 1 and 2. Among studies which measured T using immunoassay (Table 1), three studies undertaken in predominantly middle-aged men did not find any association of T with incidence of CVD events (Smith et al. 2005; Arnlov et al. 2006; Vikan et al. 2009). Of studies measuring E2 using immunoassay, one found that higher E2 was associated with lower incidence of CVD events (Arnlov et al. 2006), but another study in older men found that higher E2 was associated with increased risk of stroke (Abbott et al. 2007). The use of immunoassays for E2 in these studies is a limitation that needs to be considered. In a large population-based cohort of older

Table 1 Cohort studies examining associations between sex hormones measured using immunoassay and cardiovascular events in middle-aged and older men

Study author and year	Size (n of men)	Follow-up (year)	Age (year)	Results
Smith et al. (2005)	2,512	16.5	45–59	482 deaths and 192 fatal and 128 nonfatal IHD events. Higher cortisol/T ratio associated with IHD deaths and IHD events in age- but not multivariable adjusted analyses
Arnlov et al. (2006)	2,084	10	56	386 had first cardiovascular event. Higher total E2 at baseline associated with lower incidence of CVD events. T not associated
Abbott et al. (2007)	2,197	≤7	71–93	124 had first stroke. Baseline E2 in top quintile (≥125 pmol/L) associated with higher risk, total T not associated
Vikan et al. (2009)	1,318	9.1	59.6	146 men had first ever MI. No association of total or free T or total E2 with incident MI
Yeap et al. (2009)	3,443	3.5	≥70	First stroke or TIA occurred in 119 men. Total and free T in the lowest quartiles (<11.7 nmol/l and <222 pmol/l) predicted increased incidence of stroke or TIA
Hyde et al. (2011)	3,637	5.1	70–88	618 men experienced IHD event. Higher LH associated with incident IHD
Haring et al. (2013)	254	5, 10	75.5	No associations of baseline total T or total E2 with incident CVD events
Soisson et al. (2013)	495; 146	4	>65	495 controls, 146 men with incident CHD or stroke. Total T in lowest and highest quintiles associated with CHD or stroke

IHD ischemic heart disease, *CHD* coronary heart disease, *MI* myocardial infarction, *CVD* cardiovascular disease, *TIA* transient ischemic attack. Total T, DHT, and E2 were measured by immunoassay; free or bioavailable T and free E2 were calculated

Table 2 Cohort studies examining associations between sex hormones measured using mass spectrometry and cardiovascular events in middle-aged and older men

Study author and year	Size (n of men)	Follow-up (year)	Age (year)	Results
Ohlsson et al. (2011)	2,416	5	69–81	485 CVD events. Men with total T[a] in highest quartile (≥19 mol/L) had lower risk of CVD event. E2 was not associated
Shores et al. (2014b)	1,032	9	76	436 men had a cardiovascular event. Total T[b] not associated with cardiovascular events, DHT <1.7 or >2.6 nmol/L associated
Shores et al. (2014a)	1,032	10	76	114 men had ischemic stroke. Total T[b] not associated with stroke, DHT <1.7 or >2.6 nmol/L associated
Yeap et al. (2014b)	3,690	6.6	70–89	Incident MI occurred in 344 men, stroke in 300. T[c], DHT, and E2 not associated with MI. Higher total T (>12.6 nmol/L) or DHT (>1.34 nmol/L) associated with lower incidence of stroke
Srinath et al. (2015)	1,558	12.8	63.1	287 men had a CHD event. T[d] was not associated with incidence of CHD events

CVD cardiovascular disease, *MI* myocardial infarction, *CHD* coronary heart disease. Total T, DHT, and E2 were measured by mass spectrometry

[a]T and E2 assayed using gas chromatography-mass spectrometry (GC-MS)

[b]T and DHT assayed using liquid chromatography-tandem mass spectrometry (LC-MS)

[c]T, DHT, and E2 assayed using LC-MS

[d]T assayed using LC-MS

men, total or free T in the lowest quartile of values predicted an increased incidence of stroke or transient ischemic attacks (Yeap et al. 2009), while higher LH was associated with incidence of ischemic heart disease (IHD) events (Hyde et al. 2011). A smaller study in older men found no association of baseline T or E2 with incident CVD events, but another study also in older men reported T in the lowest and highest quintiles to be associated with CVD events (Soisson et al. 2013) suggesting a U-shaped association.

Cohort Studies Using Mass Spectrometry for Sex Steroids

When recent large cohort studies which have measured sex steroids using mass spectrometry are considered (Table 2), the results are more consistent. In the osteoporotic fractures in men (MrOS) study in Sweden, there were 2,416 men aged 69–81 years at baseline (Ohlsson et al. 2011). During a 5-year follow-up, 485 cardiovascular events including hospitalizations and deaths from coronary and cerebrovascular events occurred. The risk of experiencing a cardiovascular event was 30 % lower in men with higher total T (T ≥19 nmol/L: highest quartile vs. other

men, hazard ratio [HR] 0.70, 95% confidence interval [CI] 0.56–0.88). The Cardiovascular Health Study (CHS) involved 1,032 men aged 66–97 years (Shores et al. 2014a, b). During a 9-year follow-up, 436 men experienced a cardiovascular death or nonfatal myocardial infarction or stroke. T was not associated with this composite outcome, but DHT was with higher risk for concentrations (<1.7 or >2.6 nmol/L) (Shores et al. 2014b). When the 114 men who experienced an ischemic stroke were analyzed, total T was not associated with that outcome, but DHT was <1.7 or >2.6 nmol/L (Shores et al. 2014a). In an updated analysis from the Western Australian Health in Men Study (HIMS) involving 3,690 men aged 70–89 years at baseline, there were 644 hospital admissions or deaths due to myocardial infarction ($N = 344$) or stroke ($N = 300$) during a 6.6-year follow-up (Yeap et al. 2014b). In multivariate analyses adjusting for age and other cardiovascular risk factors, T, DHT, and E2 were not associated with incident MI. By contrast, higher T or DHT was associated with lower incidence of stroke. For men with T in the highest quartile of values (≥15.8 nmol/L) compared to the lowest (≤9.8 nmol/L), the risk of stroke was almost halved (fully adjusted HR 0.56, 95 % CI = 0.39–0.81). A similar result was found for DHT (highest quartile ≥1.8 nmol/L vs. lowest quartile ≤0.9 nmol/L, HR 0.57, 95 % CI 0.40–0.81) (Yeap et al. 2014b). The results for calculated free T paralleled those for total T. E2 was not associated with stroke. In an analysis from the Atherosclerosis Risk in Communities (ARIC) study involving 1,558 men aged on average 63.1 years, 287 coronary heart disease (CHD) events occurred during a 12.8-year follow-up (Srinath et al. 2015). In that study lower T was associated with adverse cardiovascular risk factors, but not with incidence of CHD events.

Interpretation of Cohort Studies with the Outcome of CVD Events

Allowing for the diversity of cohort studies using different assay methodologies, drawn from different populations, and analyzing varying end points relating to CVD, several conclusions can be drawn. Firstly, when considering the cohort studies based on the use of immunoassays for sex steroids (Table 1), there is little evidence that T is associated with incidence of MI per se, but there is evidence for an association of low total or free T with incidence of stroke and transient ischemic attack (Yeap et al. 2009). When considering cohort studies where sex steroids were measured accurately using mass spectrometry-based methods, neither the CHS nor the ARIC studies found any association of lower T with coronary or cardiovascular events (Shores et al. 2014b; Srinath et al. 2015). The two largest cohort studies, MrOS and HIMS, measured both T and E2 by mass spectrometry and did show positive results for T but not E2. In the case of MrOS, men with high total T had a lower risk of CVD events (Ohlsson et al. 2011), while in the case of HIMS, men with higher total or free T had a lower risk of stroke but not of MI (Yeap et al. 2014b). Therefore, lower circulating T is a biomarker for increased incidence of stroke, and this may drive associations of lower T with CVD events as a whole. This association appears to be most robust in studies of older men and may be absent in studies focused on or containing large proportions of middle-aged or even younger men. Thus, a

demarcation by age may be present: in younger and middle-aged men, lower T may be associated with adverse cardiovascular risk factors (e.g., Yeap et al. 2014c; Srinath et al. 2015) rather than incidence of CVD, while in older men, lower T or DHT is associated with increased incidence of CVD perhaps manifesting as stroke more prominently than MI (Ohlsson et al. 2011; Yeap et al. 2014b). While the association of DHT with CVD events in the CHS analyses remain to be confirmed (Shores et al. 2014a, b), the data from HIMS (Yeap et al. 2014b) confirms a role for lower DHT as a biomarker for increased incidence of stroke. Of note, androgens (T, DHT) appear to be informative biomarkers in this context, but estrogens (E2, when measured accurately with mass spectrometry) do not appear to be associated with CVD risk in men (Ohlsson et al. 2011; Yeap et al. 2014b).

Androgens, All-Cause and Cause-Specific Mortality

Cohort Studies Using Immunoassays for Sex Steroids

Longitudinal cohort studies examining the association of sex hormones at baseline with the outcome of mortality are summarized in Tables 3 and 4. In general, studies which measured T using immunoassay have reported associations of lower T with higher mortality (Table 3). These include cohort and case-control studies (Shores et al. 2006; Khaw et al. 2007; Laughlin et al. 2008; Vikan et al. 2009; Menke et al. 2010; Haring et al. 2010; Hyde et al. 2012). Several studies have reported contrasting or equivocal results or implicated other anabolic hormones in addition to T (Araujo et al. 2007; Maggio et al. 2007; Haring et al. 2013). Overall, the majority of these studies implicate lower T as a biomarker for mortality risk, albeit the studies are heterogeneous and causality remains to be proven (Araujo et al. 2011). While low T might predispose to dying, it can also be argued that underlying ill-health could result in both low T and increased mortality risk. Of note, one study found that higher E2 predicted mortality (Szulc et al. 2009), but another study suggested that lower E2 was associated with risk of mortality from CVD (Menke et al. 2010). Therefore, the relationship of E2 to mortality risk in men cannot be clearly defined from these contrasting reports.

Cohort Studies Using Mass Spectrometry for Sex Steroids

Cohort studies in which the relationship between baseline sex steroids assayed using mass spectrometry and the outcome of mortality was studied are summarized in Table 4. In the analysis from MrOS, there were 3,014 men aged on average 75 years at baseline, with 383 deaths occurring over a 4.5-year follow-up (Tivesten et al. 2009). Compared to men with total T in the lowest quartile (Q) of values ($\leq$11.7 nmol/L), those with T in the second, third, and highest quartiles had lower risk of dying from any cause (Q2 11.7–15.2 nmol/L, HR 0.71, 95 % CI 0.53–0.96; Q3 15.2–19.2 nmol/L, HR 0.55, 95 % CI 0.39–0.76; Q4 $\geq$ 19.3 nmol/L 0.59, 95 %

Table 3 Cohort studies examining associations between sex hormones measured using immunoassay and mortality in middle-aged and older men

Study author and year	Size (n of men)	Follow-up (year)	Age (year)	Results
Shores et al. (2006)	858	4.3	≥40	208 deaths. Men with two or more low T levels (total T <8.7 nmol/l or free T <0.03 nmol/l) had higher mortality
Khaw et al. (2007)	825 and 1,489	≤10	40–79	825 deaths, 1,489 controls. Total T inversely related to mortality from all causes, CVD, and cancer
Araujo et al. (2007)	1,686	15.3	40–70	395 deaths. Higher free T associated with higher IHD mortality. Equivocal association of lower DHT with IHD mortality
Maggio et al. (2007)	410	6	≥65	126 deaths. Combination of bioavailable T, insulin-like growth factor-I, and dehydroepiandrosterone sulfate in lowest quartiles associated with higher mortality
Laughlin et al. (2008)	794	11.8	50–91	538 deaths. Total T in the lowest quartile (<8.4 nmol/L) predicted increased mortality from all causes and from CVD and respiratory causes
Vikan et al. (2009)	1,568	≤13	59.6	395 deaths (130 from CVD and 80 from IHD). Free T in the lowest quartile (<158 pmol/l) predicted higher overall mortality, total T not associated
Szulc et al. (2009)	782	10	≥50	Higher total E2 predicted increased mortality after the third year
Menke et al. (2010)	1,114	18	≥20	103 deaths, 42 from CVD. Difference between 90th and 10th percentiles for free T associated with overall and CVD mortality in first 9 years of follow-up. Difference for total E2 associated with CVD mortality
Haring et al. (2010)	1,954	7.2	20–79	195 deaths. Total T <8.7 nmol/L associated with increased all-cause and CVD mortality and cancer death
Hyde et al. (2012)	3,637	5.1	70–88	605 deaths, 207 from CVD. Lower free T (100 vs. 280 pmol/L) predicted all-cause and CVD mortality
Haring et al. (2013)	254	5, 10	75.5	Higher baseline total T associated with lower 5-year but not 10-year mortality risk. E2 not associated

IHD ischemic heart disease, *CVD* cardiovascular disease. Total T, DHT, and E2 were measured by immunoassay; free T and free E2 were calculated

Table 4 Cohort studies examining associations between sex hormones measured using mass spectrometry and mortality in middle-aged and older men

Study author and year	Size (n of men)	Follow-up (year)	Age (year)	Results
Tivesten et al. (2009)	3,014	4.5	75	383 deaths. Total T[a] and E2 levels in the lowest quartiles predicted mortality. Risk of death nearly doubled in men with low levels of both total T and E2
Yeap et al. (2014a)	3,690	7.1	70–89	974 deaths, 325 from IHD. Optimal total T[b] (9.8–15.8 nmol/L) predicted lower all-cause mortality. Higher DHT (>1.3 nmol/L) predicted lower IHD mortality. E2 was not associated with mortality
Pye et al. (2014)	2,599	4.3	40–79	147 deaths. Presence of sexual symptoms, total T[c] <8 nmol/L, and free T <220 pmol/L associated with mortality
Shores et al. (2014b)	1,032	9	76	777 deaths. Total T[d] not associated with mortality, DHT <1.0 nmol/L was associated
Srinath et al. (2015)	1,558	12.8	63.1	347 deaths, 29 from CHD. Total T[e] not associated with all-cause or CHD mortality

IHD ischemic heart disease, *CHD* coronary heart disease. Total T, DHT, and E2 were measured by mass spectrometry; free T was calculated

[a]T and E2 measured using gas chromatography-mass spectrometry (GC-MS)

[b]T, DHT, and E2 measured using liquid chromatography-tandem mass spectrometry (LC-MS)

[c]T measured using GC-MS

[d]T and DHT assayed using LC-MS

[e]T assayed using LC-MS

CI = 0.42–0.83) (Tivesten et al. 2009). Interestingly, in that study comparable associations were seen for higher calculated free T and total E2 with lower all-cause mortality, but no significant associations for were seen for T or E2 with cardiovascular mortality. In the mortality analysis from HIMS, there were 3,690 men aged 70–89 years at baseline with 974 deaths occurring over a median of 7 years (Yeap et al. 2014a). Compared to men with total T in the lowest quartile (<9.8 nmol/L), those with total T in the middle two quartiles had lower all-cause mortality (Q2 9.8–12.5 nmol/L, HR 0.82, 95 % CI 0.69–0.98; Q3 12.6–15.8 nmol/L, HR 0.78, 95 % CI 0.65–0.94), with no difference seen in mortality for men with total T in the highest quartile. Similarly, midrange DHT was associated with lower all-cause mortality (DHT Q3 1.3–1.8 nmol/L, HR = 0.76, 95 % CI 0.63–0.91). Of note, higher DHT was associated with lower mortality from IHD (compared to DHT

in the lowest quartile ≤0.9 nmol/L: Q3 1.3–1.8 nmol/L, HR 0.58, 95 % CI 0.42–0.82; Q4 >1.8 nmol/L, HR 0.69, 95 % CI 0.50–0.96) (Yeap et al. 2014a). E2 was not associated with either all-cause or IHD mortality in HIMS. In the European Male Aging Study (EMAS) involving 2,599 community-dwelling men aged 40–79 years with 147 deaths occurring during a 4.3-year follow-up, there was no significant association of quintiles of either total or free T with all-cause or CVD-related mortality; instead, men with sexual symptoms and T <8 nmol/L had increased risk of death from any cause (Pye et al. 2014). In an analysis from the CHS involving 1,032 men aged 66–97 years followed for 9 years during which 777 deaths occurred, neither total nor free T was associated with all-cause or CVD-related mortality, although a curvilinear association of DHT with all-cause mortality was noted (Shores et al. 2014b).

Interpretation of Cohort Studies with the Outcome of All-Cause and Cause-Specific Mortality

It is interesting that MrOS and HIMS have provided significant and generally concordant results, which contrast to an extent with the findings of EMAS and CHS. The EMAS cohort spans a larger age range; therefore, with the inclusion of more middle-aged men and the shorter period of follow-up, there were fewer outcome events ($N = 147$) which likely reduced the statistical power of the proportional hazards regression to detect associations of baseline T with mortality (Pye et al. 2014). The ARIC study had only 29 cases of deaths due to CHD (Srinath et al. 2015). The CHS, while smaller, had longer follow-up and accumulated a large number of outcome events which paralleled marked attrition of the cohort as a whole (777 deaths in 1,032 men, 75 % cumulative mortality) (Shores et al. 2014a). This begs the question whether there is an optimal time frame during the life of a prospective cohort study during which mortality analyses may be at their most informative. Early on, limited numbers of outcome events may reduce the power of statistical approaches. With extended follow-up, attrition of a cohort through advancing age, or "drift" of biochemical variables away from the initial baseline value, might impair its ability to define the utility of biomarkers for outcomes of interest. MrOS and HIMS are large studies in older men, with sufficient outcome events during defined periods of follow-up to enable robust longitudinal analyses using proportional hazards regression for the outcome of death from any cause ($N = 383$ and $N = 974$, respectively) and, in the case of HIMS, for the outcome of IHD mortality ($N = 325$) (Tivesten et al. 2009; Yeap et al. 2014a). The MrOS analyses were adjusted for age, MrOS site, body mass index (BMI), physical activity, and current smoking, with supplementary analyses excluding prevalent cancer, CVD, or diabetes which did not alter the direction of the results (Tivesten et al. 2009). In HIMS, comprehensive adjustments were made for factors that could plausibly confound associations with mortality. Models were age adjusted, with subsequent additional adjustment for education, smoking, BMI, and waist/hip ratio (WHR); then for hypertension, dyslipidemia, diabetes, and creatinine; and finally for

history of cancer or existing CVD (Yeap et al. 2014a). The results of MrOS indicate higher total T and E2 are predictors of reduced all-cause mortality in older men, while HIMS found that an optimal rather than high total T was associated with the longest survival in older men with no association of E2 (Tivesten et al. 2009; Yeap et al. 2014a). It is possible that the larger number of outcome events in the HIMS analysis could allow better definition of an underlying U-shaped association of T with all-cause mortality. The larger number of outcome events and the comprehensive adjustment for potential confounders might better illustrate the absence of an association of E2 with the same outcome. In HIMS, results for calculated free T mirrored those for total T. The fully adjusted model containing total T was further explored by means of sequential incorporation of SHBG and LH, which showed that total T was an independent predictor (Yeap et al. 2014a). Finally, the findings from HIMS indicate that in older men, higher DHT is a biomarker for lower risk of death from IHD (Yeap et al. 2014a), but not incidence of MI (Yeap et al. 2014b). Thus, higher circulating DHT may represent a survival or resilience factor following an IHD event.

The Testosterone Controversy

Randomized Controlled Trials and Meta-analyses

There have been randomized controlled trials (RCTs) of T supplementation which have shown cardioprotective effects. In a study of 46 men with stable angina, transdermal T supplementation over 12 weeks reduced exercise-induced myocardial ischemia (English et al. 2000). In a smaller study of 15 men with angina, intramuscular long-acting depot T over 12 months reduced time to ischemia on exercise testing (Mathur et al. 2009). In a study of 87 older men with diabetes, oral T undecanoate over 12 weeks reduced the frequency of angina attacks (Cornoldi et al. 2010). However, these studies were either of limited duration (English et al. 2000; Cornoldi et al. 2010) or small in terms of number of participants (Mathur et al. 2009). Therefore, the publication of the Testosterone in Older Men with Mobility Limitations (TOM) trial caused considerable controversy (Basaria et al. 2010). The TOM trial recruited men who were 65 years of age or older, with a total T of 3.5–12.1 nmol/L or a free T of $<$173 pmol/L, with evidence of limitations in mobility. Participants were randomized to receive 100 mg daily transdermal T or placebo for 6 months. The trial was stopped prematurely after 209 of the target sample of 252 men had been randomized, due to an excess of cardiovascular adverse events being reported in the T arm (Basaria et al. 2010). Men in the TOM trial were 74 years old on average, and half had preexisting CVD. This contrasts with a comparable study in which 274 men aged 65 years or more who were frail or intermediate-frail with total T $\leq$12 nmol/L or free T $\leq$250 pmol/L were randomized to 50 mg daily transdermal T or placebo for 6 months (Srinivas-Shankar et al. 2010). That study was completed successfully with no signal for adverse cardiovascular events, finding that T supplementation improved muscle strength and physical

function. Of note, those effects were not maintained at 6 months' posttreatment (O'Connell et al. 2011). Neither of these RCTs (nor for that matter any preceding RCTs) were designed to examine cardiovascular events as prespecified outcomes, utilizing reporting of adverse events (Basaria et al. 2010; Srinivas-Shankar et al. 2010). Therefore, this sparked a renewed interest in meta-analyses of T RCTs to clarify whether or not T supplementation was associated with cardiovascular adverse events, as summarized in Table 5. Meta-analyses completed prior to the

Table 5 Meta-analyses of randomized controlled trials (RCTs) of T supplementation examining associations of T therapy with cardiovascular adverse events

Study characteristics				Results	
Study author and year	N of RCTs	N active	N placebo	Adverse signal	No adverse signal
Haddad et al. (2007)	30	808	834		No significant difference in odds ratio for any cardiovascular adverse event or for MI
Fernandez-Balsells et al. (2010)	51	2,716			No significant difference for all-cause mortality, coronary bypass surgery, or MI
Xu et al. (2013)	27	2,994		T associated with increased risk cardiovascular-related event (OR 1.54, 95 % CI = 1.09–2.18)[a]	
Ruige et al. (2013)	10 (>100 participants)	1,289	848		No significant difference in cardiovascular adverse events
Corona et al. (2014)	75	3,016	2,448		No association of T supplementation with cardiovascular risk. For MACE, OR = 1.01 (95 % CI 0.57–1.77)

MI myocardial infarction, *MACE* major adverse cardiovascular events, *OR* odds ratio, 95 % *CI* confidence interval. Unless otherwise specified, meta-analyses were conducted using random effects models

[a]Fixed effects model

publication of the Basaria and Srinivas-Shankar studies did not find any significant difference in risk of cardiovascular adverse events in T compared with placebo recipients (Haddad et al. 2007; Fernandez-Balsells et al. 2010). A meta-analysis by Xu et al. including both the Basaria and Srinivas-Shankar trials reported an increased risk of cardiovascular-related events reported as cardiac, cardiovascular, or vascular disorders associated with T (Xu et al. 2013). However, a meta-analysis by Ruige et al. also including these two RCTs and focusing on larger RCTs found no significant difference in cardiovascular adverse events with T compared to placebo (Ruige et al. 2013). More recently, Corona et al. conducted a meta-analysis including 75 trials with 3,016 men receiving T and 2,448 men receiving placebo for a mean duration of 34 weeks (Corona et al. 2014). In that meta-analysis, T was not associated with excess cardiovascular adverse events. When major cardiovascular events (cardiovascular death, nonfatal acute MI and stroke, acute coronary syndromes, and/or heart failure reported as serious adverse events) were considered, there was no difference in risk between the T and placebo recipients (Corona et al. 2014). Therefore, meta-analyses of RCTs in general have not found T supplementation to be associated with excess cardiovascular adverse effects.

Observational Studies of T Prescriptions

Definitive RCTs powered for the outcome of cardiovascular events would be logistically difficult to conduct, needing large numbers of men to be randomized and treated for an extended duration of time. Therefore, retrospective studies of health care or insurance databases have been conducted to examine associations of T prescriptions with outcomes, as summarized in Table 6. These studies have substantive limitations, including their observational nature and the absence of randomization, limited clinical data regarding the indications for T treatment and T concentrations during treatment, and, in specific studies, concern over the analytical approaches utilized. Shores et al. in a study of male veterans in the United States with a baseline total T $\leq$8.7 nmol/L found those who were prescribed T supplementation experienced 39 % lower mortality compared to those who did not receive T (Shores et al. 2012). Muraleedharan in a study of men with type 2 diabetes in the United Kingdom with baseline total T $\leq$10.4 nmol/L found that those men who received T supplementation also experienced lower mortality compared to those who did not (Muraleedharan et al. 2013). These studies suggested a potential benefit of T supplementation, albeit care is needed with their interpretation due to their observational nature and potential for selection bias in the prescription of T (Wu 2012). In a controversial study involving a different cohort of male veterans in the United States who underwent coronary angiography and had total T $\leq$10.4 nmol/L, Vigen et al. reported that men prescribed T had a 29 % higher risk of adverse outcomes defined as the composite of death or hospitalization for MI or ischemic stroke (Vigen et al. 2013). However, looking at the actual data, 1,233 men started T, of whom 67 died, 23 had MIs, and 33 had strokes: a total of 123 men or 10.1 %. There were 7,486 men who did not receive T, of whom 681 died, 420 had MIs, and 486 had

Table 6 Retrospective studies of T prescribing in middle-aged and older men which have examined associations of T supplementation with cardiovascular events and mortality

Study characteristics				Results	
Study author and year	N	Follow-up (year)	Age (year)	Favor no T	Favor T
Shores et al. (2012)	1,031	3.4	62.1		Male veterans with total T ≤8.7 nmol/L, T prescribed in 398. T supplementation associated with lower mortality
Muraleedharan et al. (2013)	581	5.8	59.5		Men with type 2 diabetes, 238 with total T ≤10.4 nmol/L. T supplementation associated with lower mortality
Vigen et al. (2013)	8,709	2.3	63.4	Male veterans who had coronary angiography and total T ≤10.4 nmol/L. T prescription associated with increased risk of death, MI, or stroke	
Finkle et al. (2014)	55,593	90 days	54.4	Men prescribed T. Higher rate of nonfatal MI in 90 days following prescription compared to preceding 1 year	
Baillargeon et al. (2014)	6,355; 19,065	4.1; 3.3	≥66		Men prescribed T versus matched nonusers. T prescription not associated with increased risk of MI. For men at highest risk, T associated with reduced risk of MI

MI myocardial infarction

strokes: a total of 1,587 or 21.2 %. Therefore, the actual observed rate of adverse outcome events in men prescribed T was half that of the men who were not prescribed T, but the direction of the results was reversed by a complex statistical model drawing critical comment (Traish et al. 2014). The subsequent published erratum acknowledged incorrect classification of a number of men in the original

analysis and identified 100 women who needed to be excluded (Vigen et al. 2014). A retrospective study by Finkle et al. examined the risk of nonfatal MI in the 90 days following a prescription of T with the preceding 3-month period using a large healthcare database (Finkle et al. 2014). That study reported an increased risk for men aged 65 years and older or men under the age of 65 who have a prior history of CVD, based on a relatively small number of incident events and a low absolute event rate. That study lacked a suitable control group relying instead on comparison with men receiving phosphodiesterase therapy, and there were no results reported for fatal events or for events occurring after 90 days (Finkle et al. 2014). By contrast, the more recent study by Baillargeon et al. assessed a national sample of Medicare beneficiaries 66 years age or older and found that T treatment was not associated with risk of MI (HR 0.84, 95 % CI 0.69–1.02) (Baillargeon et al. 2014). In that study, in men at the highest risk of MI based on a prognostic score, T treatment was associated with lower risk of MI (HR 0.69, 95 % CI 0.53–0.92). While all of these studies have recognized limitations, the contrasting results illustrate the need for definitive prospective randomized controlled trials to clarify whether T supplementation in middle-aged or older men would reduce or increase the risk of CVD. Pending further studies, a conservative approach could be adopted for older men with preexisting CVD presenting for management of androgen deficiency.

Testosterone and Dihydrotestosterone: Biomarkers or More?

These studies have highlighted the topical nature of the T debate. There is evidence from well-conducted epidemiological studies that demonstrate the association of lower circulating T with a range of poorer health outcomes in older men including prevalent CVD (e.g., Yeap et al. 2012a), incident CVD events particularly stroke (e.g., Ohlsson et al. 2011; Yeap et al. 2014b), and mortality (e.g., Tivesten et al. 2009; Yeap et al. 2014a). Lower circulating DHT is also associated independently with incidence of stroke (Yeap et al. 2014b) and with higher mortality from IHD (Yeap et al. 2014a). These studies have adjusted for age and other conventional risk factors for CVD; thus, the associations reported cannot be accounted for by these means. Therefore, lower circulating T and DHT are robust biomarkers for incidence of CVD, particularly stroke, and in the case of DHT for IHD mortality. While the multivariate analyses of longitudinal data are consistent with a role for T or DHT to influence risk of CVD events, caution is required before inferring causality. To prove causation requires demonstration of an effect of T in RCTs to reduce cardiovascular risk, and this is where the evidence gap lies. From RCTs which were not powered for the prespecified outcomes of MI or stroke, but reported cardiovascular adverse events, one found increased such adverse events with T treatment (Basaria et al. 2010), while another did not (Srinivas-Shankar et al. 2010). The studies of T prescriptions in men cannot substitute for an adequately powered RCT and showed conflicting results. Thus, lower endogenous circulating T is a robust biomarker for CVD events and mortality and lower DHT for stroke and IHD

mortality, but further studies are needed to establish whether or not these are in fact potentially remediable risk factors for CVD events and thereby mortality.

Potential Applications to Prognosis, Other Diseases, or Conditions

Application of Androgens as Biomarkers

The knowledge that lower T and DHT, but not E2, are biomarkers for poorer CVD-related outcomes and mortality is of interest. Here, a clear distinction needs to be drawn between men who have low T (or DHT) and who are androgen deficient, for example, due to pituitary or testicular disease, and those who are not androgen deficient but have T (or DHT) within the reference range but at the lower end of the distribution. Men who are androgen deficient, who have symptoms and signs of androgen deficiency and unequivocally low early morning T concentrations confirmed by repeat measurements using accurate assays, should be considered for replacement therapy (Bhasin et al. 2010). If middle-aged or older men are found to have circulating T or DHT in the low-normal range, then these results indicate an increased risk for poorer health outcomes in keeping with the role of lower T and DHT as biomarkers. Bearing in mind that the role of T supplementation in men with low-normal circulating levels in the absence of pathological androgen deficiency remains under debate (Cunningham and Toma 2011), interventions in these men should focus on appropriate encouragement of healthy lifestyle behaviors and attention to modification of established risk factors for cardiovascular disease such as hypertension and hypercholesterolemia (Yeboah et al. 2015).

Implications of Potential Role for Lower Circulating T and DHT as Risk Factors

Additional studies are needed to clarify whether androgen supplementation would alter the risk of stroke or other CVD-related events in men with low-normal circulating T or DHT who do not have pituitary or testicular disease. This would require large-scale RCTs powered to detect effects of T on cardiovascular events, which to date have not been performed. The United States-based Testosterone Trials has recruited 788 men aged $\geq$65 years, with self-reported sexual dysfunction, diminished vitality and/or mobility limitations, and two early morning T concentrations averaging $<$9.5 nmol/L, with neither $>$10.4 nmol/L (Cunningham et al. 2015). The intent of the Testosterone Trials was to test whether 1 year's intervention with transdermal T supplementation in these men would improve outcomes relating to physical function, sexual function, vitality, cognitive function, plaque volume, bone density, and anemia (https://clinicaltrials.gov, identifier NCT00799617, accessed May 2015). While the primary outcomes and the safety data from that study will be of great interest, even this RCT will not be powered for

the outcome of CVD events. The Australian Testosterone for the Prevention of Type 2 Diabetes in Men at High Risk (T4DM) trial is a multicenter RCT currently in progress seeking to recruit 1,488 men with impaired glucose tolerance or newly diagnosed type 2 diabetes, with total T $\leq$14 nmol/L (https://www.anzctr.org.au, identifier ACTRN12612000287831, accessed May 2015). T4DM will examine the efficacy of T treatment together with a lifestyle program in comparison to a lifestyle program alone, to normalize glucose tolerance in those with newly diagnosed type 2 diabetes or prevent progression to type 2 diabetes in those with impaired glucose tolerance. Even with a larger recruitment target and the 2-year duration of intervention, T4DM will not be powered for the outcome of cardiovascular events. Nevertheless, the metabolic and other outcomes and the safety data accruing from that trial will be of great interest. The stage is set for additional studies to explore mechanistic pathways by which T might influence cardiovascular risk and ultimately to clarify whether or not T supplementation could preserve health in the increasing population of older men.

Summary Points

- Circulating concentrations of testosterone (T) are lower in older compared to younger men.
- Lower circulating T is associated with a range of poorer health outcomes in older men.
- T is metabolized to the more potent androgen dihydrotestosterone (DHT).
- Both lower T and DHT concentrations are associated with higher risk of stroke.
- Lower DHT concentrations are associated with increased mortality from ischemic heart disease.
- Interventional studies of T therapy have not been powered for the outcome of cardiovascular events or mortality.
- Meta-analyses of RCTs in general have not found T supplementation to be associated with excess cardiovascular adverse effects.
- Reduced T and DHT are robust biomarkers for heart disease and related mortality.
- Additional studies are required to determine causality and clarify the effect of T supplementation on health in older men.

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Leukotrienes as Biomarkers of Cardiovascular Disease

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_42

Abstract

Myocardial infarction and stroke are major causes of morbidity and mortality and result from an underlying atherosclerosis of the coronary and cerebrovascular vasculature. Atherosclerotic plaques are a site of lipid accumulation and chronic inflammation. There is a need for novel biomarkers to predict an individual's cardiovascular risk, and several inflammatory biomarkers have been explored for their prognostic value. Leukotrienes are lipid mediators of inflammation, which are formed in atherosclerotic lesions and participate in the atherosclerosis process. The local production of leukotrienes leads to high levels in atherosclerotic plaques, whereas circulating levels are negligible and difficult to measure. Ex vivo stimulation of leukocytes reflects the leukotriene synthesizing capacity and the leukotriene B_4 levels released from granulocytes in response to calcium inophore are associated with echographic measures of carotid artery vascular remodeling. Urinary leukotriene E_4 is a validated biomarker of asthma, and is increased in coronary artery disease. Salivary levels of leukotriene B_4 were recently associated with vascular stiffness and subclinical atherosclerosis. Leukotriene measures have in addition been associated with several cardiovascular risk factors, such as smoking, diabetes, obesity, and obstructive sleep apnea. The present chapter reviews the available literature using these different approaches for evaluating leukotrienes as biomarkers for cardiovascular disease.

Keywords

Atherosclerosis • Ageing • Inflammation • Leukotriene • Lipoxygenase • Obstructive sleep apnea • Pulse Wave Velocity • Saliva • Urinary biomarkers • Vascular stiffness

Abbreviations

5-LO	5-lipoxygenase
BAL	Broncho-alveolar lavage fluid
BMI	Body mass index
CABG	Coronary artery by-pass grafting
COPD	Chronic obstructive pulmonary disease

$cPLA_2$	Cytosolic phospholipase A_2
EBC	Exhaled breath condensates
eGFR	Estimated glomerular filtration rate
EIA	Enzyme immunoassay
FLAP	5-LO activating protein
GCF	Gingival crevicular fluid
GSH	Glutathione
hsCRP	High sensitivity C-reactive protein
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDL	Low density lipoprotein
LT	Leukotrienes
PCI	Percutaneous coronary intervention
γ-GT	γ-glutamyl transpeptidase

Introduction

Biomarkers of Cardiovascular Diseases

Cardiovascular disease remains the leading cause of death despite major advances in diagnostics and treatment. The major underlying pathology is atherosclerosis (Table 1), which is triggered by accumulation of cholesterol-containing low-density lipoprotein (LDL) particles in the arterial wall leading to immune activation and the recruitment of inflammatory cells (Hansson 2005; Libby et al. 2011; Bäck and Hansson 2015). The resulting atherosclerotic plaques may remain silent for a long time before plaque destabilization occurs, leading to plaque rupture and occlusion o,f for example, cerebrovascular and coronary arteries, causing myocardial infarction and stroke, respectively.

Traditional cardiovascular risk factors exhibit a high predictive value on a population level, but fail to fully predict individual risk (Hoefer et al. 2015). The identification of subjects at increased risk for plaque rupture and resulting cardiovascular events is of particular interest to select patients who would benefit from preventive actions and medical treatments. In this context, a role for inflammatory biomarkers such as high sensitivity C-reactive protein (hsCRP) has emerged as independent risk factors for acute coronary events (Libby et al. 2002), further underlining the role of inflammation in the atherosclerosis process. As stated in a

Table 1 Key facts of atherosclerosis

Atherosclerosis is characterized by lipid retention and immune activation within the vascular wall, and release of inflammatory mediators from the atherosclerotic plaques
Destabilization of atherosclerotic plaques leads to plaque rupture, thrombosis and vessel occlusion resulting in cardiovascular events, such as myocardial infarction and stroke
Biomarkers for the prediction of cardiovascular events are of substantial interest for identifying subjects who would benefit from preventive treatment and/or interventions

This table lists the key facts of atherosclerosis and the context of biomarkers

recent *Position Paper* from the European Society of Cardiology, biomarkers with causal involvement may be more valuable for risk stratification (Hoefer et al. 2015) and may also be useful for identifying novel therapeutic targets and used in drug efficacy evaluation (Bäck and Hansson 2015).

Leukotriene Biosynthesis

Arachichidonic acid is released from cell membrane phospholipids by calcium-dependent activation of the intracellular cytosolic group *IV*A phospholipase A_2 ($cPLA_2$) and is the substrate for the formation of leukotrienes (LT; Fig 1). Metabolism by means of the 5-lipoxygenase (5-LO) enzyme, in conjunction with a 5-LO activating protein (FLAP), will yield the epoxide LTA_4, which serves as precursor for LT synthesis. Whereas the enzyme LTA_4 hydrolase leads to formation of LTB_4, the conjugation of LTA_4 with glutathione (GSH) will yield LTC_4. Subsequently, LTs are transported to the extracellular space where LTC_4 shares its subsequent metabolism with GSH by means of γ-glutamyl transpeptidase (γ-GT) and dipeptidase that cleaves the peptide bonds of the LTC_4 side chain forming LTD_4 and LTE_4, respectively (Fig 1).

Importantly, 5-LO is highly expressed in myeloid cells, e.g., granoluocytes, macrophages, and mast cells, leading to local LT biosynthesis at sites of inflammation, which in some cases also involves transcellular metabolism of LTA_4 in for

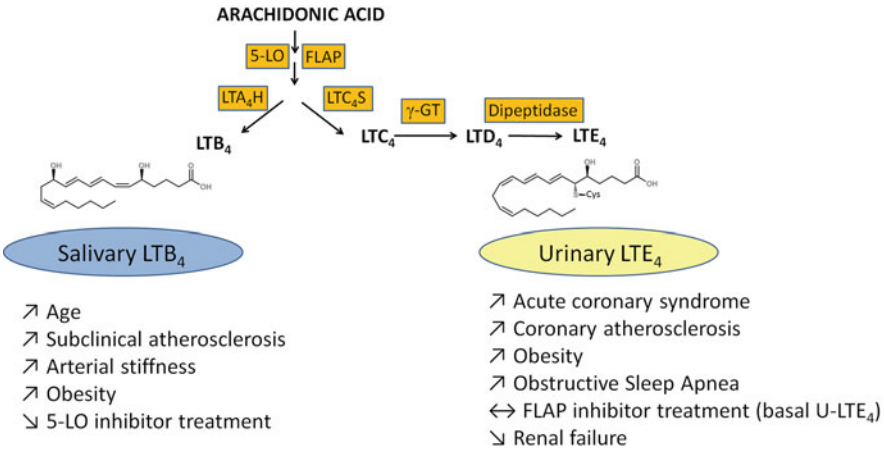

Fig. 1 Leukotriene (LT) biosynthesis through the 5-lipoxygenase (5-LO) pathway of arachidonic acid metabolism, and possible biomarker applications for salivary LTB_4 (Gaber et al. 2006; Bäck et al. 2007b; Labat et al. 2013) and urinary LTE4 (Carry et al. 1992; Allen et al. 1993; Dahlen et al. 1997; Stanke-Labesque et al. 2009; Rafnsson et al. 2013; Bäck et al. 2014a) in cardiovascular disease and cardiovascular risk factors. Abbreviations: FLAP, 5-LO Activating Protein; γ-GT, γ-glutamyl transpeptidase; LTA_4H, LTA_4 hydrolase; LTC_4S, LTC_4 synthase

example endothelial cells, vascular smooth muscle cells, platelets and macrophages. In addition, 5-LO expression is regulated by promoter methylation (Katryniok et al. 2010), and LT synthesis from arachidonic acid may be induced in non-myeloid cells through epigenetic mechanisms (Nagy and Bäck 2012).

Taken together, this local leukotriene production at sites of cardiovascular inflammation can lead to high leukotriene concentrations at their sites of action, which may not necessarily be reflected in their circulating levels. The latter raises several challenges in the exploration of leukotrienes as biomarkers for cardiovascular disease, which will be the focus of the present chapter.

The Role of Leukotrienes in Cardiovascular Disease

Leukotrienes exert potent actions on inflammatory reactions, being active at nanomolar concentrations at specific G-protein coupled leukotriene receptors (Bäck et al. 2011) expressed on several target cells in atherosclerotic lesions (Bäck et al. 2014b). Leukotrienes exert diverse proinflammatory effects with implications for atherosclerosis development and cardiovascular disease, inducing leukocyte recruitment and activation, smooth muscle cell proliferation, and endothelial dysfunction (Bäck and Hansson 2006; Bäck 2009). In addition, the leukotriene pathway has been linked to cardiovascular calcification in aortic valve stenosis (Nagy et al. 2011). The degree of calcification can alter the biomechanical properties of the vascular wall (Bäck et al. 2013; Kwak et al. 2014) and is also of importance since microcalcifications in the fibrous cap might be associated with plaque rupture (Otsuka et al. 2014). Importantly, leukotriene receptor antagonists used in the treatment of asthma have been associated with a decreased risk of stroke and myocardial infarction (Ingelsson et al. 2012) and antileukotriene has been evoked as putative therapeutics in cardiovascular prevention (Bäck and Hansson 2015). Given this implication of the LT pathway as a causal factors in cardiovascular diseases (Bäck 2009), there is an increasing interest to monitor leukotrienes as biomarkers in cardiovascular disease (Hoefer et al. 2015).

Leukotriene Measurements: Methodological Considerations

The analytical challenge for leukotriene quantitation lies in the detection of small amounts of leukotrienes in different biological fluids, e.g., urine, saliva or cell supernatants. Several analytical methods have been described including enzyme immunoassays (EIA) or chromatography tandem mass spectrometry methods.

EIA is an antibody-based method, the validity of which depends on the specificity of the antibody used. Although easy to perform, the main limitation of this strategy is the cross-reactivity of the antibody with other potential interfering compounds in the matrix, despite a previous purification step. This point is a major concern when analyzing urinary-LTE_4. In addition, EIA does not allow the separation of

enantiomers or diastereoisomers, and provides, for example, an overestimation of LTB_4 concentration in supernatant of challenged cells.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is a more specific method based on a chromatographic separation followed by detection of the compound of interest on its mass/charge ratio. Following an enrichment step by solid phase extraction (Hardy et al. 2005) or on-line extraction (Armstrong et al. 2009) these methods offer greater specificity, precision and accuracy and allow for example the separation of LTB_4 and its isomers 6-trans-LTB_4 and 6-trans-12-epi LTB_4 (Stanke-Labesque et al. 2012) thanks to the chromatographic step.

When compared to EIA, the concentrations of LTE_4 measured by LC-MS/MS are lower in urine from patients with asthma highlighting the greater specificity of tandem mass spectrometry (Armstrong et al. 2009). There is hence a huge need to organize international interlaboratory proficiency testing programs to standardize the quantitation of leukotrienes.

Human Vascular Leukotriene Production

Ex vivo studies of vascular specimens have confirmed that leukotriens are locally produced within the human vascular wall. This has, for example, been studied in the pulmonary vasculature (Piper et al. 1988) and perfused and ventilated human lungs (Kiss et al. 2000). Importantly, atherosclerotic lesions exhibit an increased leukotriene formation compared with healthy vessels, as demonstrated by studies of human vascular segments derived from both carotid endarterectomies (De Caterina et al. 1988), and human coronary arteries (Piomelli et al. 1987).

Recent methodological advances have opened up for selective lipidomic analysis, which has revealed that lipoxygenase metabolites are the predominant arachidonic acid products formed in carotid atherosclerotic lesions, compared with cyclooxygenase products (Liu et al. 2013). In addition, human abdominal aortic aneurysms and stenotic aortic valves are also sites of local leukotriene formation (Houard et al. 2009; Nagy et al. 2011; Kochtebane et al. 2013). Taken together, these studies provide the rationale for exploring leukotrienes as biomarkers of cardiovascular disease.

Plasma and Serum Leukotriene Measurements

Analyses of blood samples withdrawn at the site of obstructive atherosclerotic coronary lesions have revealed undetectable LT levels (Brezinski et al. 1992). In contrast, LTC_4 and LTD_4 (but not LTE_4) were detectable in samples taken at the site of the coronary lesion immediately after the balloon inflation during percutaneous coronary intervention (PCI) (Brezinski et al. 1992). Although the latter study provides compelling evidence that plaque rupture may be the stimulus triggering appearance of these mediators in plasma, no peripheral blood samples were

withdrawn at the time of the angioplasty and the extrapolation to systemic plasma measures of leukotrienes remains unknown.

Another study reported increased LT plasma levels (in samples withdrawn from the femoral artery) in 19 patients with myocardial infarction compared with 12 healthy controls. These elevated levels persisted for more than 1 week and had returned to values comparable to those observed for control subjects at 1 month from the myocardial infarction (Takase et al. 1996). This observation may indicate that leukocyte activation in myocardial infarction persists, but it remains unclear whether the results either reflect the atherosclerotic plaque rupture or the myocardial damage following the ischemia.

Several initial studies measured plasma levels of leukotrienes during coronary artery bypass grafting (CABG), and generated somewhat variable results. Despite pronounced coronary atherosclerosis, plasma levels of LTB_4 and LTC_4 are negligible in patients undergoing CABG (Gimpel et al. 1995). However, the perioperative extracorporeal circulation during CABG may increase plasma LTs to detectable levels, especially after release of the aortic cross-clamp (Jansen et al. 1991; Gimpel et al. 1995; Denizot et al. 1999). In contrast, one study reported unchanged LTC_4 concentrations during CABG in both the radial artery and the coronary sinus (Bengtson et al. 1989).

These reported either low or undetectable LT levels in plasma samples are contrasted by high levels in serum sample (Houard et al. 2009), raising the notion that serum LT levels may be due to ex vivo LT production from, for example, neutrophil granulocytes during the coagulation process in the tube. In support of the latter, serum LTB_4 levels are associated with the time from blood sampling to centrifugation (Houard et al. 2009), hence emphasizing the limits of measuring systemic circulation LT levels as biomarkers.

LT Release from Ex Vivo Stimulated Leukocytes

Since plasma LT levels do not necessarily reflect the local production at the site of the vascular inflammation, another approach is to isolate leukocytes from human blood followed by ex vivo stimulation with calcium ionophore, which activates 5-LO by means of increased intracellular calcium. Given that leukocytes are the predominant source of LTs biosynthesis, ex vivo stimulation of circulating leukocytes may reflect the leukocyte capacity of LT formation.

Using this approach, an increased LTB_4 formation in subjects with a history of myocardial infarction has been described (Helgadottir et al. 2004). In the latter study, myocardial infarction patients exhibiting the highest stimulated leukocyte LTB_4 production were carriers of the described 5-LO haplotype, which was associated with increased cardiovascular risk (Helgadottir et al. 2004), supporting stimulated LT release as an appropriate measure of LT-producing capacity. Furthermore, the levels of LTB_4 released from calcium ionophore-stimulated granulocytes, derived from subjects with obstructive sleep apnea, exhibited a significant association with

carotid artery measures of vascular remodelling (Lefebvre et al. 2008), supporting the use of stimulated LT release as biomarker for subclinical atherosclerosis (Stanke-Labesque et al. 2014). Finally, LTB_4 formation is increased in granulocytes collected after the extracorporeal circulation during CABG, with the elevation being persistent during the first 24 postoperative hours, and was suggested to have a significant role in the postoperative outcome (Gadaleta et al. 1994).

Taken together, evaluating LT-synthesizing capacity in ex vivo stimulated leukocytes has been explored as biomarkers of genetic variations in LT-producing enzymes (Helgadottir et al. 2004), subclinical atherosclerosis (Lefebvre et al. 2008) and in thoracic surgery (Gadaleta et al. 1994). However, the extensive experimental work involved in the sample preparation may limit the use of ex vivo stimulated leukocytes for the evaluation of LTs as biomarkers for cardiovascular disease.

Urinary LTE_4

Validation of Urinary-LTE_4 as a Biomarker

Injection of radiolabeled LTC_4 or LTE_4 to healthy volunteers revealed that these LTs appear as LTE_4 in the urine within 1–2 h after infusion (Orning et al. 1985; Sala et al. 1990; Maclouf et al. 1992). In contrast, only low levels of LTB_4 metabolites can be detected in urine samples after LTB_4 injection (Berry et al. 2003). Subsequent studies established the use of urinary LTE_4 as a biomarker for asthma and allergen-provoked bronchoconstriction (Dahlen et al. 1997; Dahlen and Kumlin 1998; Balgoma et al. 2013). There are in contrast only a limited number of studies applying urinary LTE_4 as cardiovascular biomarker.

Urinary-LTE_4 in Myocardial Infarction and CABG

In acute myocardial infarction an increase in urinary LTE_4 has been reported, decreasing to control values 3 days after ischemia (Carry et al. 1992). As discussed above, while this approach does not allow distinguishing plaque rupture from myocardial damage as the cause of increased LTs, the latter study suggests that urinary LTE_4 decreased to concentrations similar to controls at the third day after the ischemic event. This is in contrast to a study of patients undergoing CABG who had significantly elevated urinary LTE_4 compared with healthy controls (Allen et al. 1993). Whether the apparent differences between those studies were due to more extended atherosclerosis in the CABG candidates cannot be concluded from the available data. Nevertheless, taken together those studies suggest that urinary LTE_4 may be used for both the evaluation of baseline values in atherosclerosis and reflect changes in acute coronary syndromes and myocardial ischemia. The latter notion is supported by the further increase in urinary LTE_4 observed after CABG

surgery, with a peak of urinary LTE_4 at the second postoperative day (Allen et al. 1993).

Limitations of Urinary LT Measures

In a recent study, urinary LTE_4 did not correlate with either macro- or microvascular endothelial function in a cohort of diabetes patients with microalbuminuria (Rafnsson and Bäck 2013). In contrast, urinary LTE_4 levels were significantly decreased with impaired renal function, and multivariate analysis revealed the estimated glomerular filtration rate (eGFR) as an independent predictor of urinary LTE_4 concentrations in these patients (Rafnsson and Bäck 2013). These data imply that renal function should be considered when studying urinary biomarkers, especially when evaluating cardiovascular risk, for which renal function may be a significant confounder. However, in a cohort with normal renal function, no significant associations between urinary LTE_4 and eGFR were detected (Bäck et al. 2014a), hence supporting the use of this biomarker in the absence of renal failure.

Another limitation of urinary LTE_4 has emerged from clinical studies of LT synthesis inhibitors. Whereas the allergen-induced increase in urinary LTE_4 concentrations is effectively inhibited by the FLAP antagonist BAYx1005/DG031, the basal prechallenge urinary LTE_4 concentrations were unaltered by this treatment (Dahlen et al. 1997). In addition, a study evaluating effects on cardiovascular biomarkers reported an unexpected increase in urinary LTE_4 in subjects treated with BAYx1005/DG031 (Hakonarson et al. 2005). Those studies hence question the use of urinary LTE_4 for the evaluation of pharmacological LT synthesis efficacy in cardiovascular clinical trials.

Salivary LTB_4

In the exploration of biomarkers for pulmonary disease, LT levels have been measured in exhaled breath condensates (EBC). Studies in thoracic surgery have shown that while LTB_4 levels in EBC are unaltered during CABG, significant changes were observed in pulmonary lobectomy (Moloney et al. 2004), supporting that biomarkers in EBC may more accurately predict pulmonary compared with cardiovascular diseases. However, studies of LT concentrations in EBC revealed the possibility of EBC contamination with LTs derived from the oral cavity (Gaber et al. 2006).

The notion of oral LTs as biomarkers is supported by studies of periodontitis, in which gingival crevicular fluid (GCF) can be sampled at the site of periodontal inflammation. LT concentrations are increased in GCF from periodontitis patients (Tsai et al. 1998; Bäck et al. 2006). Interestingly, GCF concentrations of LTs are also increased in subjects with carotid artery atherosclerotic plaques on echographic

examination (Bäck et al. 2006), providing a first piece of evidence of an association between oral LTs and cardiovascular disease.

Since GCF sampling requires dental intervention, GCF may not be a suitable matrix for cardiovascular biomarker evaluations. However, also saliva contains remarkably high levels of LTB_4 (Gaber et al. 2006; Bäck et al. 2007b). Furthermore, in contrast to urinary LTE_4 (Dahlen et al. 1997; Hakonarson et al. 2005), a reduction of basal LT production by 5-LO inhibition can be monitored by measuring salivary LT levels (Gaber et al. 2007), further reinforcing the accuracy of salivary LTB_4 as a possible biomarker.

In a cohort of 259 subjects, salivary LTB_4 exhibited a significant association with echographic measures of subclinical atherosclerosis, as defined by the carotid artery intima media thickness (Labat et al. 2013). Furthermore, the vascular stiffness was evaluated by means of pulse wave velocity. In a multivariate analysis, salivary LTB_4 was an independent predictor for increased arterial stiffness (Labat et al. 2013). Since both the intima media thickness and pulse wave velocity are prognostic markers for cardiovascular outcome, the associations with these measures provide a first indication for the potential use of salivary LTB_4 as a biomarker for cardiovascular disease. Given that sampling of unstimulated whole buccal saliva is a simple and non-invasive procedure reinforces the suitability of salivary LTB_4 for biomarker evaluation in large cardiovascular cohort studies.

LTs as Biomarkers in Relation to Cardiovascular Risk Factors

When studying leukotriene formation in cardiovascular disease it is also important to address how known cardiovascular risk factors affect LT biomarker concentrations, both in terms of confounding factors, but also as a potential causal factor and source of LTs.

Age

The normal wear and tear of aging will lead to a progressive deterioration of cardiovascular structures which may contribute to cardiovascular disease development. Moreover, aging is associated with an accumulation of cardiovascular risk factors (Thomas et al. 2001), and needs to be taken into consideration when evaluating cardiovascular biomarkers. Salivary LTB_4 is significantly increased in older subjects (Bäck et al. 2007b), whereas ex vivo stimulated LTB_4 release (Lefebvre et al. 2008; Stanke-Labesque et al. 2012) and urinary LTE_4 (Stanke-Labesque et al. 2009) appears less dependent on age. It should however be pointed out that the significant associations of salivary LTB_4 with carotid artery intima media thickness and body mass index (BMI) persisted in an age-adjusted analysis (Labat et al. 2013).

Smoking and Chronic Obstructive Pulmonary Disease (COPD)

Increased LT levels have been reported in EBC (Carpagnano et al. 2003), sputum (Keatings et al. 1996), and bronchoalveolar lavage fluid (BAL) (Zijlstra et al. 1992), derived from smokers compared with nonsmokers. LT concentrations in BAL were in addition correlated to the number of granulocytes, suggesting that increased LT levels in pulmonary samples may reflect increased neutrophilic inflammation in the lungs induced by smoking. Likewise, urinary LTE_4 is increased in smokers compared with non-smokers, and correlates with the number of cigarettes smoked (Fauler and Frolich 1997). In the latter context, also underlying pulmonary pathologies may affect the urinary LTE_4 response to smoking (Gaki et al. 2007).

In contrast, GCF and saliva LT levels do not differ between smokers and nonsmokers (Bäck et al. 2006, 2007b), suggesting that the biomarker sampling fluid may be crucial for the detection of smoking-induced LT production. Finally, with the reservation of plasma LT measures previously addressed (cf. *supra*), no difference in plama-LTB_4 concentrations between smokers and nonsmokers have been reported in a study of 61 healthy subjects (McKarns et al. 1995).

Assessed by means of ex vivo stimulated LTB_4 release, subjects with COPD exhibit an increased LT production (Mitsunobu et al. 2001; Santus et al. 2005). Importantly, in addition to sharing smoking as common risk factor, COPD has emerged as an independent risk factor for cardiovascular disease (Nishiyama et al. 2010; Yin et al. 2014). The association of COPD with an increased cardiovascular risk has been suggested to relate to both similar risk factors and to similar pathophysiological mechanisms, in which LT-induced inflammatory circuits may be involved (Bäck 2008), hence reinforcing the interest of evaluating LTs as biomarkers when assessing cardiovascular risk associated with smoking and COPD.

Diabetes

Urinary LTE_4 is increased in type 1 diabetic patients with poor metabolic control (Hardy et al. 2005) and intense glycemic control decreased urinary LTE_4 in type 1 diabetes but not in type 2 diabetes (Boizel et al. 2010). In overweight subjects, urinary LTE_4 is significantly higher in subjects with high fasting plasma glucose (Bäck et al. 2014a), supporting that diabetes and insulin resistance should be considered as possible confounders in the exploration of urinary LTE_4 as cardiovascular biomarker.

Obesity

The association between LT production and obesity was initially demonstrated in subjects with obstructive sleep apnea (Stanke-Labesque et al. 2009) and was subsequently reported in asthmatics (Giouleka et al. 2011) and in children with sleep

disordered breathing (Shen et al. 2011). Further exploration in a cohort of obese subjects revealed a significant correlation of urinary LTE_4 with the waist to hip ratio (Bäck et al. 2014a). Finally, the association of LTs with obesity and waist-to-hip ratio has also been observed for salivary LTB_4 (Labat et al. 2013).

The association of LTs not only with BMI but also waist circumference is of particular clinical interest, since abdominal obesity is a predominant cardiovascular risk factor in obesity. Adipose tissue may represent an active site of leukotriene formation in experimental studies (Bäck et al. 2007a). In addition, FLAP is expressed in human adipose tissue, with higher levels in abdominal subcutaneous fat derived from obese compared with lean subjects (Kaaman et al. 2006).

Obstructive Sleep Apnea

Obstructive sleep apnea is characterized by recurrent episodes of nocturnal upper airway obstruction leading to chronic intermittent hypoxia, which is a potent proinflammatory stimulus. Increased LT levels in urine (Stanke-Labesque et al. 2009; Shen et al. 2011), EBC (Goldbart et al. 2006) and ex vivo stimulated leukocytes (Lefebvre et al. 2008; Stanke-Labesque et al. 2012) have been reported in subjects with obstructive sleep apnea, and correlated with different measures of disease severity. The increased cardiovascular risk associated with obstructive sleep apnea has been well established (Levy et al. 2012), and leukotrienes have been implicated as possible causal factor for the accelerated atherosclerosis associated with obstructive sleep apnea (Stanke-Labesque et al. 2014).

Potential Applications to Prognosis, Other Diseases or Conditions

As outlined above, LTs are increased in different cardiovascular diseases (Table 2). Given that these potent lipid mediators of inflammation are produced locally at their site of action, systemically measured levels may not necessarily reflect increased levels at sites of inflammation, such as atherosclerotic lesions. Plasma and serum measures of LTs cannot be recommended based on the existing literature. As an alternative, assessment of leukotriene synthesis from ex vivo stimulated leukocytes provides a reliable measure of LT synthesizing capacity, but its use may be limited by the experimental preparations needed. Urinary LTE_4 has been validated as a biomarker in asthma, but only limited data are available for this biomarker in cardiovascular disease. Despite certain precautions needed, such as taking renal function into considerations, urinary LTE_4 is an interesting and feasible approach for assessing LTs as biomarkers in cardiovascular disease. However, LTB_4 concentrations cannot be measured in the urine, and for this mediator, saliva measures may represent an alternative. Finally, we draw the attention to important confounders to take into consideration when assessing LTs as biomarkers, such as age, smoking, and obesity, as well as comorbidities in terms of COPD, diabetes, and obstructive sleep apnea. In conclusion, although the limited available studies on LTs as biomarkers in

Table 2 Leukotrienes as biomarkers of cardiovascular disease

	Plasma	Stimulated Leukocytes	Urine	Saliva	Other
Acute Coronary Syndrome	↑[1]		↑[2]		
CABG	↑[3–5], ↔[6]	↑[7]	↑[8]		↔ in EBC[9]
PCI	↑[10]				
History of AMI		↑[11]			
Carotid Artery Echography Measures		↑[12]		↑[13]	↑ in GCF[14]
Vascular Stiffness				↑[13]	
Cardiovascular Risk factors					
Age		↔[12, 15]	↔[16, 17]	↑[18]	
Smoking	↑[19]		↑[19, 20],↔[20, 21]	↔[13]	↑ in EBC[22], sputum[23] and BAL[24], ↔ in GCF[14]
Diabetes			↑[25]		
Obesity		↔[12, 15]	↑[16, 26]	↑[13]	
Obstructive Sleep Apnea		↑[12, 15]	↑[16]		↑ in EBC[27]

This table lists the key studies of leukotrienes as biomarkers for different measures of cardiovascular disease, and of different cardiovascular risk factors. Abbreviations: *CABG* coronary artery by-pass grafting, *EBC* exhaled breath condensates, *PCI* percutaneous coronary intervention, *AMI* acute myocardial infarction, *GCF* gingival crevicular fluid, *BAL* bronco-aleveolar lavage
References: [1]Takase et al. 1996; [2]Carry et al. 1992; [3]Jansen et al. 1991; [4]Gimpel et al. 1995; [5]Denizot et al. 1999; [6]Bengtson et al. 1989; [7] Gadaleta et al. 1994; [8]Allen et al. 1993; [9]Moloney et al. 2004; [10]Brezinski et al. 1992; [11]Helgadottir et al. 2004; [12]Lefebvre et al. 2008; [13]Labat et al. 2013; [14]Bäck et al. 2006; [15]Stanke-Labesque et al. 2012; [16]Stanke-Labesque et al. 2009; [17]Rafnsson and Bäck 2013; [18]Bäck et al. 2007b; [19]Fauler and Frolich 1997; [20]Gaki et al. 2007; [21]McKarns et al. 1995; [22]Carpagnano et al. 2003; [23]Keatings et al. 1996; [24]Zijlstra et al. 1992; [25]Hardy et al. 2005; [26]Bäck et al. 2014; [27]Goldbart et al. 2006

cardiovascular disease (Table 2) are promising, there is a need for standardization of LT measurements to reflect and detect increased LT formation in cardiovascular diseases.

Summary Points

- This chapter focuses on the lipid mediators of inflammation, leukotrienes, and their role as biomarkers of cardiovascular disease, especially atherosclerosis.
- Leukotrienes are lipid mediators of inflammation, formed locally at sites of inflammation by means of 5-lipoxygenase metabolism of arachidonic acid.

- Local production of leukotrienes leads to high levels in atherosclerotic plaques, whereas circulating levels are negligible and difficult to measure.
- Ex vivo stimulation of leukocytes reflects the leukotriene synthesizing capacity and the leukotriene B_4 levels released from granulocytes in response to calcium inophore are associated with echographic measures of carotid artery vascular remodeling.
- Urinary leukotriene E_4 is a validated biomarker of asthma, and is increased in coronary artery disease, but some precautions are needed when applying urinary leukotriene E_4 as cardiovascular biomarker.
- Salivary levels of leukotriene B_4 were recently associated with vascular stiffness and sublinical atherosclerosis.
- Leukotriene measures have in addition been associated with several cardiovascular risk factors, such as smoking, diabetes, obesity, and obstructive sleep apnea.
- There is a need for standardization of LT measurements to reflect and detect increased LT formation in cardiovascular diseases.

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Plasma 8-Isoprostane as a Biomarker and Applications to Cardiovascular Disease

21

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_31

Abstract

Increased oxidative stress has been increasingly recognized as a contributing factor in several pathological conditions. Isoprostanes – a class of substances which has been revealed as potential biomarkers of level of oxidative stress in vivo – have been indicated as mediator of cardiovascular diseases and responsible for increasing cardiovascular risk. Therefore, plasma 8-isoprostane arises as a compound derived from lipid peroxidation able to indicate the endogenous oxidative stress with a large body of evidence to be associated with cardiovascular disease. The great majority of studies have focused their works on atherosclerotic disease, probably due to severe clinical complications deriving from the disease as well as the wide evidences linking the oxidative stress to conditions that underlie the atherosclerotic process such as endothelial dysfunction and inflammation. On the other hand, hypertension, stroke, heart failure, and non-cardiovascular conditions – such as pulmonary and neurological diseases as well as cancer and liver affections – have also acquired prominence in this concern. Almost all studies have supported the hypothesis that the evaluation of oxidative stress compounds may represent additional prognostic predictors in such conditions as well as targets for the development of novel strategies of pharmacological therapy. However, it is important to note that the association observed in the studies does not imply a causal relationship.

Keywords

8-Isoprostane • Oxidative stress • Lipid peroxidation • Cardiovascular disease • Atherosclerosis

Abbreviations

8-Iso-PGF2α	8-Iso-prostaglandin F2 alpha
8-OHdG	8-Hydroxy-20-deoxyguanosine
ACEI	Angiotensin-converting enzyme inhibitor
ARR	Aldosterone-renin ratio
AT1 receptor	Angiotensin type I receptor
BP	Blood pressure
CAC	Coronary artery calcification
CAD	Coronary artery disease
CPAP	Continuous positive airway pressure
DCs	Dendritic cells
GC-MS	Gas chromatography-mass spectrometry
hsCRP	High-sensitivity C-reactive protein
LDLs	Low-density lipoproteins
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate oxidase
NAFLD	Nonalcoholic fatty liver disease
NO	Nitric oxide
OSAS	Obstructive sleep apnea syndrome
ox-LDL	Oxidized LDL

RHTN	Resistant hypertension
ROS	Reactive oxidative species
sICAM-1	Soluble intercellular adhesion molecule 1
SR hypertension	Salt-resistant hypertension
SS hypertension	Salt-sensitive hypertension
TNF-α	Tumor necrosis factor-alpha
sVCAM-1	Soluble vascular cell adhesion molecule 1

Key Facts of Oxidative Stress

- It is important to point out that reactive oxygen species – the compounds that may participate as trigger of oxidative stress – are continually produced in the organism underlying the normal metabolic processes and interactions with environmental stimuli.
- Under physiologic conditions, the biological system has a wide range of antioxidant defenses protecting against the adverse effects of the production of reactive oxygen species.
- In pathological conditions, there is an overproduction of reactive oxygen species, and/or the antioxidant defenses can be overwhelmed.
- Diseases can be followed by the imbalance between oxidant and antioxidant components in favor of oxidants which characterize the oxidative stress status.
- Increased oxidative stress has been implicated in the pathophysiology of several human diseases – including atherosclerosis, hypertension, and pulmonary and neurological diseases, such as asthma and Alzheimer disease – with important considerations of biological structure impairment and, consequently, the health damage.
- Isoprostanes – the compounds derived from the attack of reactive oxygen species on cell lipid membranes – have been indicated as potential biological markers of indirect measurement of oxidative stress in the organism.
- Isoprostanes have been studied in biological fluids - mainly measured in urine and blood samples - and are still confined to research setting due to the high costs and specialized staff, although it has been revealed of great interest to advance in the clinical routine use.

Definitions

A randomized, double-blind, placebo-controlled trial One of the study categories including subjects and that currently considered the best evidence to determine a cause-effect relationship of an intervention on a specific end point and is also designed to avoid the great majority of confounding factors.

Cardiovascular events Phenomenon commonly occurring in the heart, renal, and cerebral arteries, due to their continuous narrowing and, in a final instance, to its

occlusion. Represented mainly by the acute myocardial infarction, chronic kidney disease, stroke, angina, and transient ischemic attack.

Endothelial function Measurement of the endothelium capacity to produce vasodilator compounds, in which the nitric oxide is the most important studied substance in cardiovascular disease.

Gas chromatography-mass spectrometry A highly used technique for chemical analysis by which mixtures of chemicals may be separated, identified, and quantified. The primary condition to be analyzed by this technique is that the compound of interest must be volatile.

Isoprostanes A class of prostaglandin isomers derived by reactive oxidative species attack on lipid content of cell membranes, which have been revealed as potential biomarkers of the level of oxidative stress in vivo.

Immunoassays A biochemical technique to measure the molecules in biological liquids such as serum and urine that uses the enzymatic principle to reveal an antigen-antibody reaction.

Lipid peroxidation Nonenzymatic reaction caused by reactive oxidative species attack on lipid content of cell membranes.

Oxidative stress The status characterized by an imbalance favoring reactive oxidative species production to the detriment of antioxidant capacity of the biological system resulting in disease process.

Reactive oxidative species Current physiological compounds, which have highly reactive properties to interact with lipids and proteins of an organism and in excess may contribute to pathological conditions such as cardiovascular diseases.

Surrogate end points Outcomes of clinical studies that are not directly a main end point (such as death, stroke, or heart attack for cardiovascular outcomes). Sometimes they are markers associated with the main outcome.

Introduction

The status of oxidative stress is characterized as an imbalance favoring reactive oxidative species (ROS) production to the detriment of antioxidant capacity of the biological system (Crimi et al. 2007). Increased oxidative stress has been increasingly recognized as a contributing factor in several pathological conditions (Gopaul et al. 1995; Morrow et al. 1995; Wang et al. 1995).

The direct measurement of ROS is quite difficult, basically because of its primary condition (a highly reactive species with a consequent short life). Thus, the oxidative

stress has been measured by assessing the damage induced by ROS on specific compounds. Isoprostanes – a class of substances which has been revealed as potential biomarkers of level of oxidative stress in vivo (Morrow and Roberts 1999; Morrow 2005) – have been indicated as mediator of cardiovascular diseases, and also of some non-cardiovascular diseases, and responsible for increasing cardiovascular risk (Costa et al. 2012; Vazzana et al. 2013). Moreover, the isoprostanes have demonstrated biological effects participating through transduction mechanisms linking oxidant stress to specialized forms of cellular activation, such as platelet activation and smooth muscle cell proliferation in cardiovascular disease (Patrono and FitzGerald 1997).

The possibility of quantifying – either in vitro or in vivo – the oxidative damage by measuring oxidative stress compounds along with its rate of production has become of great interest in research and clinical settings. Formation of isoprostanes in vivo can be reliably monitored noninvasively by analytical approaches that yield sensitive and specific indexes of oxidative stress status in humans. Moreover, these compounds are biochemically stable which also contribute to designate them as reliable biomarkers (Pratico 1999). Therefore, plasma 8-isoprostane arises as a compound derived from lipid peroxidation able to indicate the endogenous oxidative stress with a large body of evidence to be associated with cardiovascular disease.

Relevance of the 8-Isoprostane Generation in the Health-Disease Process

Isoprostanes are stable end products of lipid peroxidation derived from arachidonic acid by a cyclooxygenase-independent mechanism that result from the attack of oxidative reactive species on phospholipids in the cell membranes (Morrow et al. 1990b) or in circulating low-density lipoproteins (LDLs) (Lynch et al. 1994). In addition, it has been reported that isoprostanes can be produced as a minor product of the cyclooxygenase enzyme activity (Pratico et al. 1995). Isoprostanes are present in the circulation of individuals mainly in its ester isoforms, whereas only hydrolyzed isoprostane compounds are excreted in the urine. Although a large number of end products can be generated after the reaction of reactive oxidative species on lipid membranes, the most interest of studies has focused on the F2-isoprostanes – a family of prostaglandin isomers – and, in particular, on 8-iso-prostaglandin F2 (8-iso-PGF2α) (Fig. 1).

It has been shown that F2-isoprostanes have no diurnal variations (Helmersson and Basu 1999), which contributes to reduce potential confounders for the measurement of one biomarker in routine. Moreover, F2-isoprostane levels seem to be unaltered by the lipid content of the diet (Gopaul et al. 2000), which also contribute to establish isoprostanes as a reliable biomarker for assessing oxidative stress in vivo.

The presence of detectable concentrations of isoprostanes in biological fluids such as plasma or urine indicates the continuing lipid peroxidation process despite the participation of the organism protection known as antioxidant defenses. Moreover, studies have suggested that circulating isoprostane concentrations are mainly

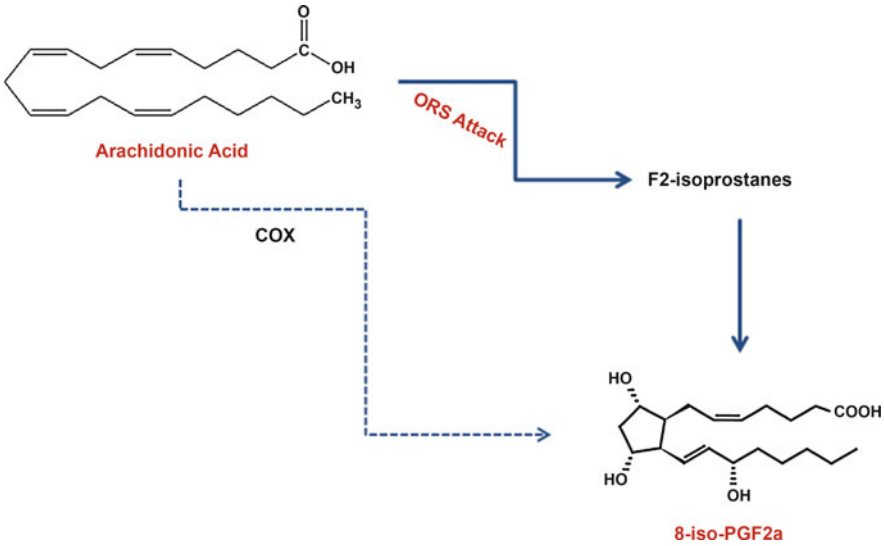

Fig. 1 The process of 8-isoprostane generation. The figure shows the formation of 8-iso-prostaglandin F2 alpha (8-iso-PGF2α) derived from lipid peroxidation, i.e., the arachidonic acid through a cyclooxygenase (*COX*)-independent mechanism is attacked by oxidative reactive species (*ORS*). Isoprostanes can be also produced as a minor product of the COX enzyme activity

dependent on its production rather than metabolism and excretion, suggesting that these compounds may truly state the level of oxidant stress in vivo (Morrow and Roberts 1999; Morrow 2005).

Suggested as the best available markers of oxidative stress, some isoprostanes – such as 8-iso-PGF2α – have biological functions with relevance for its measurement in the concern of cardiovascular disease pathophysiology (Vassalle et al. 2003) (Fig. 2). In addition, 8-iso-PGF2α has been indicated as a potent vasoconstrictor (Takahashi et al. 1992), also demonstrated in coronary arteries (Kromer and Tippins 1996), and able to induce DNA synthesis in vascular smooth muscle cells (Fukunaga et al. 1993). Finally, the great majority of studies have focused their works on atherosclerotic disease, probably due to severe clinical complications deriving from the disease, as well as the wide evidences linking the oxidative stress to conditions that underlie the atherosclerotic process such as endothelial dysfunction and inflammation.

Cardiovascular Disease

Atherosclerosis

Gross et al. have reported an association between increased concentrations of circulating 8-iso-PGF2α and coronary artery calcification (CAC) – a component of coronary artery atherosclerosis – in young healthy adults participating in the

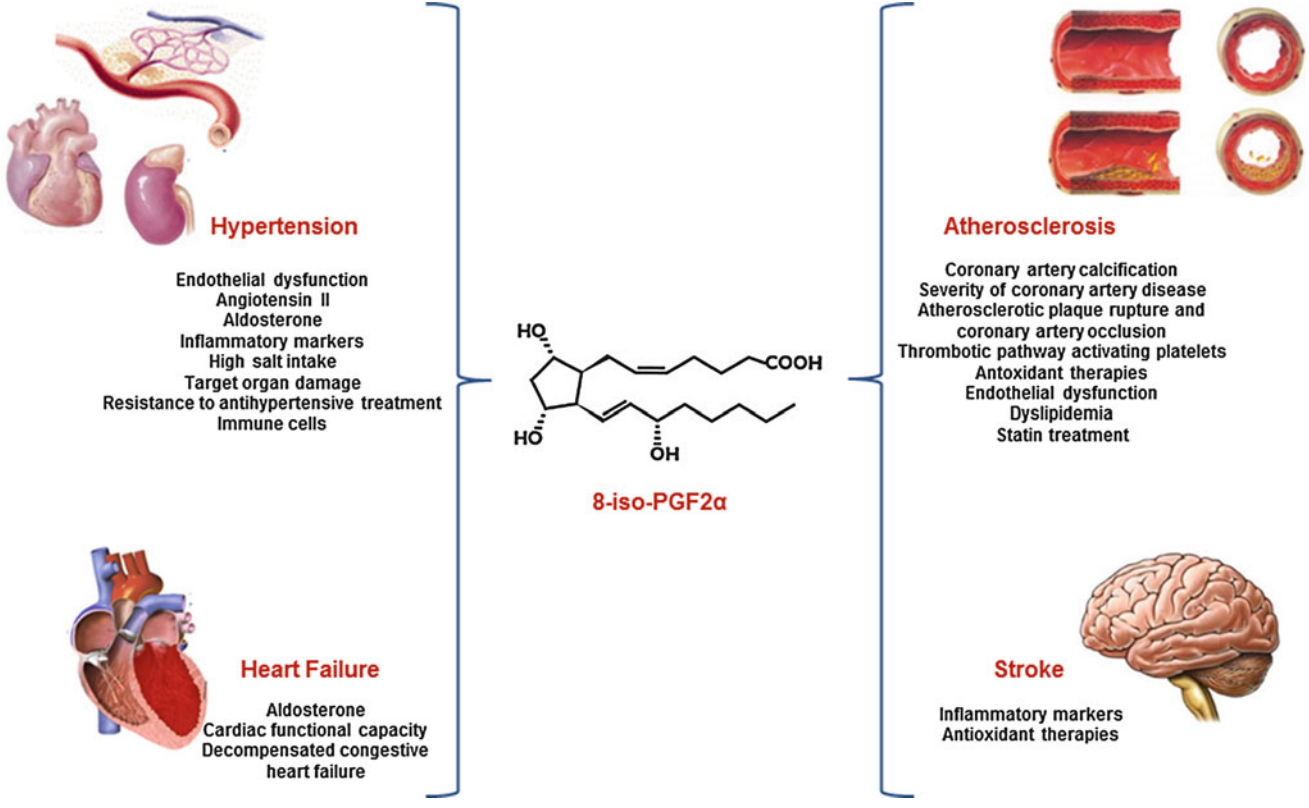

Fig. 2 Potential conditions linking the 8-isoprostane to cardiovascular disease. The figure shows the topics discussed on literature associating the marker of oxidative stress 8-iso-prostaglandin F2 alpha (8-iso-PGF2α) to the main cardiovascular diseases such as atherosclerosis, hypertension, stroke, and heart failure

Coronary Artery Risk Development in Young Adults (CARDIA) study. Plasma F2-isoprostanes were associated with CAC in both genders, independently of conventional cardiovascular risk factors and C-reactive protein. Individuals with high compared to low concentrations of 8-iso-PGF2α had the highest prevalence of CAC. Indeed, CAC is considered a strong predictor of cardiovascular events in prospective studies and may be considered as a good surrogate end point of cardiovascular disease. These findings, therefore, have confirmed an association between oxidative damage and the early stages of atherosclerotic process in humans supporting the hypothesis that oxidative compounds are involved in the early disease of atherosclerosis (Gross et al. 2005).

Clinical evidences for enhanced oxidative stress in coronary artery disease (CAD) have been reported. Patients who underwent coronary angiography to verify coronary artery atherosclerotic lesions have been assessed by concentrations of 8-iso-PGF2α in the plasma samples. It was observed that the levels of this biomarker were increased in patients with angiographically proven CAD compared to healthy control subjects. Interestingly, 8-iso-PGF2α levels also increased with severity of CAD, determined by the number of affected arteries (one-, two-, or three-coronary disease) with a luminal diameter reduction over 50 %. Still, the extent of CAD was considered an independent determinant factor affecting 8-iso-PGF2α levels. These findings also contribute to suggest a pivotal role of oxidative stress observed in the onset and progression of the atherosclerosis – especially in the coronary arteries (Vassalle et al. 2003; Wang et al. 2006). Furthermore, it was demonstrated that acute coronary syndrome subjects have increased levels of this oxidative stress associated with platelet activation compared to patients diagnosed for stable CAD which, in turn, have indicated high intensity of oxidative stress generated by atherosclerotic plaque rupture and coronary artery occlusion (Szuldrzynski et al. 2010).

A study has determined whether isoprostanes were present in human atherosclerotic lesions, where lipid peroxidation is thought to occur in vivo. A marked elevation of 8-iso-PGF2α was found in atherosclerotic plaque compared to healthy vessels in human submitted to carotid endarterectomy. The content of 8-iso-PGF2α was also detected by immunohistochemistry in lipid-rich atherosclerotic lesions predominantly associated with macrophages and smooth muscle cells. This supports the idea that the measurement of isoprostanes may provide a quantitative manifestation of oxidant stress in human atherosclerotic disease (Pratico et al. 1997).

It has been highlighted that 8-iso-PGF2α might participate as one of the mediators in some biological conditions such as linking lipid oxidation with thrombotic complications in atherosclerotic disease (Berliner et al. 1995). In this context, a study have indicated that 8-iso-PGF2α activates platelets by inducing platelet adhesion – also increasing intracellular calcium concentration – and reducing the antagonist activity of nitric oxide (NO) toward its antiadhesive and antiaggregatory effects (Venturini et al. 1992; Radomski and Moncada 1993). Thus, 8-iso-PGF2α may mediate thrombotic pathway directly activating platelets and reducing the vasodilator biological activity of functional endothelium-derived NO (Minuz et al. 1998). Increased oxidative stress process has also been suggested as a mediator for endothelial dysfunction in type II diabetic patients possibly by reducing endothelium-derived NO (Ting

et al. 1996). Patients with type II diabetes compared to healthy subjects have been investigated after taking for 1 week an oral treatment of raxofelast – a hydrophilic antioxidant vitamin E analogue (Cuzzocrea et al. 1999). After the treatment with this antioxidant drug, plasma concentrations of 8-iso-PGF2α were reduced in diabetic patients, but no significant change was observed in control individuals. In the same way, forearm blood flow response to acetylcholine – technique to assess endothelial function – was greater than baseline in patients with diabetes after use of medication improving endothelial function (Chowienczyk et al. 2000).

Indeed, the treatment with statin, but not fibrate, has been recognized to exert antioxidant effect (Giroux et al. 1993; De Caterina et al. 2002). A study has assessed the effects of simvastatin (40 mg/day) and bezafibrate (800 mg/day) treatment on total 8-iso-PGF2α patients with hypercholesterolemia without other cardiovascular risk or confounding factors. Levels of plasma 8-iso-PGF2α significantly decreased during the 6 months of the simvastatin treatment period, but it was not observed after bezafibrate treatment. Moreover, supplementation of antioxidant vitamin E reduced total 8-iso-PGF2α concentrations in simvastatin-treated patients (Desideri et al. 2003). In addition as expected, patients with dyslipidemia have presented higher 8-iso-PGF2α levels compared to non-dyslipidemia subjects (Vassalle et al. 2003). On the other hand, the effects of short-term fenofibrate treatment (160 mg/day) on oxidized LDL (ox-LDL) and 8-iso-PGF2α levels have been investigated in a sub-cohort multicenter study. The treatment significantly lowered triglycerides in the similar manner of ox-LDL and 8-iso-PGF2α levels with the greatest reductions observed in individuals with elevated baseline values (Dong et al. 2011).

Despite general acceptance of the importance of oxidative stress in atherosclerosis disease, clinical trials designed to use antioxidant supplementations have produced disappointing results disfavoring the use of these therapies (Hennekens et al. 1996; Yusuf et al. 2000; Heart Protection Study Collaborative Group 2002). A randomized, double-blind, placebo-controlled trial of beta-carotene has failed to demonstrate benefit of long-term use with supplementation on the incidence of cardiovascular disease or death from all causes in healthy men (Hennekens et al. 1996). Moreover, in patients at high risk for cardiovascular events – since they had cardiovascular disease or diabetes in addition to one other risk factor – daily treatment with vitamin E also failed to demonstrate any apparent effect on primary outcomes composite of myocardial infarction, stroke, and death from cardiovascular causes (Yusuf et al. 2000). Several explanations for these negative results emerge, including (i) the use of inappropriate antioxidants or combinations and incorrect doses of them, (ii) a short duration of treatment, or (iii) failure to initiate the supplementation earlier in the atherosclerotic process.

Hypertension

Increased oxidative stress in hypertension has been attributed to endothelial dysfunction caused by NO inactivation (Vaziri et al. 1999; Taddei et al. 2001),

generation of isoprostanes from lipid peroxidation (Pratico et al. 1998), and a direct action as vasoconstrictor or reducing vasodilator activity (Tesfamariam and Cohen 1992). Experimental studies have associated angiotensin II with increased plasma F2-isoprostanes, and also its levels have been important in the pathophysiology of hypertension by increasing and maintaining blood pressure (BP), among others, through the stimulation of oxidative stress (Romero and Reckelhoff 1999; Reckelhoff et al. 2000). Indeed, endothelial dysfunction and oxidative products are present in early events of cardiovascular disorders induced by angiotensin II (Wattanapitayakul et al. 2000).

A clinical study revealed a significant increase in 8-iso-PGF2α levels among hypertensives compared to nortensive subjects, and indicated hypertension as a determinant factor able to change the levels of the biomarker (Vassalle et al. 2003; Cottone et al. 2007). The 8-iso-PGF2α divided successively according to quartiles has shown that along with the increasing percentiles, the levels of the endothelial dysfunction parameters – such as high-sensitivity C-reactive protein (hsCRP), tumor necrosis factor-alpha (TNF-α), soluble intercellular adhesion molecule-1 (sICAM-1), and vascular cell adhesion molecule-1 (sVCAM-1) – have increased. Also, hsCRP, ICAM-1, and TNF-α were considered predictors of the oxidative stress marker, independently of BP levels (Vassalle et al. 2003; Cottone et al. 2007).

In addition, the potential antioxidant properties of antihypertensive drugs, such as angiotensin receptor antagonist, have been investigated. Irbesartan 150 mg/day was used for subjects with metabolic syndrome, and after 4 weeks of therapy, the plasma levels of 8-iso-PGF2α reduced by 15 % compared to the placebo group. In addition, interleukin-6 and plasminogen activator inhibitor-1 – markers of inflammation that are implicated in the atherosclerotic disease – were reduced by 25 % and 19 %, respectively. This improvement in inflammatory status was accompanied by increases in flow-mediated dilation of the brachial artery by 67 % after the short-term treatment with irbersartan (Sola et al. 2005). Indeed, the blockade of angiotensin type I (AT1) receptor in experimental design has demonstrated reduction of (i) nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity – an important enzyme in producing oxidizing agents found in the immune cells neutrophils, monocytes, and macrophages (Babior 2004) – as well as (ii) atherosclerotic plaque area and macrophage infiltration into subendothelial arteries, improving endothelial function (Ferrario et al. 2002). Moreover, angiotensin-converting enzyme inhibitor (ACEI) ramipril has been tested in mouse model of type II diabetes after 6 weeks of therapy. Although this drug did not affect body weight and glucose levels, the treatment reduced 8-iso-PGF2α which was followed by improvement of acetylcholine-induced endothelium-dependent vasodilation compared to untreated models. Thus, the inhibition of angiotensin II by ACEI treatment may downregulate oxidative stress-related compounds (Liang et al. 2008).

Higher levels of aldosterone – a regulating mineralocorticoid hormone of water and electrolytes balance in the body – are frequently found in subjects with hypertension and have also been indicated to mediate maladaptive cardiovascular

changes by increasing oxidative stress. An experimental study has shown that aldosterone may induce oxidative stress by the increase of plasma 8-iso-PGF2α causing cardiac and renal profibrotic effects (Iglarz et al. 2004). Furthermore, spironolactone – a mineralocorticoid receptor antagonist – has been shown to prevent cardiac fibrosis involving angiotensin II by acting through AT1 receptors (Robert et al. 1999).

The role of the salt on hypertension has contributed to conflicting results mainly due to the studied population with different hypertensive phenotypes. Because of that, several studies have denominated subjects as having salt-sensitive (SS) and salt-resistant (SR) hypertension to better understanding of the mechanisms of salt in the disease. In experimental studies involving SS hypertension, high salt intake has been associated with target organ damage by increasing oxidative stress (Atarashi et al. 1997; Somova et al. 2001; Trolliet et al. 2001). Moreover, a clinical study has shown an inappropriate lack of aldosterone suppression in response to a salt load leading to high aldosterone-renin ratio (ARR) in SS hypertensive subjects (Lim et al. 2002). In addition, the relationship between changes in 8-iso-PGF2α biomarker by salt depletion and ARR has been indicated, which suggests that oxidative stress may enhance in cases where the salt cannot suppress aldosterone (Laffer et al. 2006). Ultimately, these findings supported the idea that target organ damage of SS hypertension could be mediated by aldosterone-induced oxidative stress (Fukunaga et al. 1993; Rocha et al. 2000).

Resistant hypertension – a specific condition of hypertension – is known as the BP that remains above goal (140/90 mmHg) in spite of the concurrent use of three or more antihypertensive drugs of different classes in optimal doses (Calhoun et al. 2008). Recently, a cross-sectional study has found that 8-iso-PGF2α levels were markedly higher in resistant compared to mild to moderate hypertensive subjects. Also, 8-iso-PGF2α levels were inversely associated with endothelial function in RHTN group, even after the adjustment for potential confounders such as age, gender, body mass index, type II diabetes, smoking habits, levels of aldosterone and LDL cholesterol, and systolic BP. It was demonstrated that the association between oxidative stress and endothelial dysfunction might involve a BP-independent mechanism (de Faria et al. 2014) (Fig. 3). Moreover, experimental study has investigated the role of oxidative damage in the genesis of hypertension. Initially, the group of researchers has demonstrated that models of hypertension induced by angiotensin II increased reactive oxidative species production in antigen-presenting dendritic cells (DCs) and, consequently, released cytokines, such as interleukins 6, 1β, and 23. DCs from angiotensin II-infused mice also promoted T cell proliferation and production of cytokines interleukin-17, interferon gamma, and TNF-α. In addition, it has been shown that hypertension modulates DC gene expression and that many of these gene changes were dependent on oxidative stress. Therefore, this study has evidenced a new mechanism for the genesis of hypertension, which suggests a huge potential to be explored in the development of drugs for the treatment of this condition.

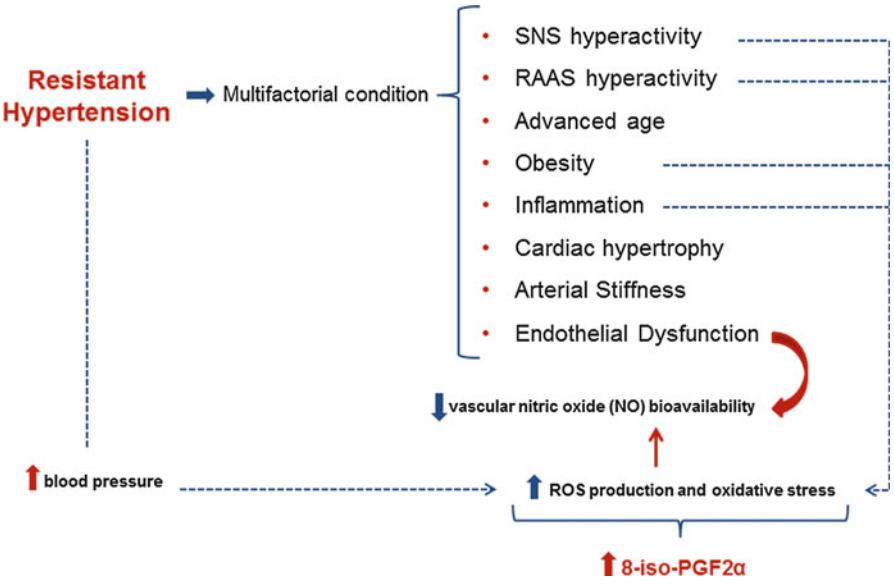

Fig. 3 Pathophysiology of resistant hypertension. The figure shows the multifactorial pathophysiology of resistant hypertension associating the increased levels of 8-iso-prostaglandin F2 alpha (8-iso-PGF2α) with impairment of endothelial function. However, although the oxidative stress may predict endothelial dysfunction, it is unknown whether this is a cause or a consequence of increased BP and other conditions such as sympathetic nervous (*SNS*) and renin-angiotensin-aldosterone (*RAAS*) systems hyperactivity, obesity, and inflammation

Stroke

Oxidative stress has been associated with stroke (Rao and Balachandran 2002; Liu 2003). A study has compared the nutritional status and levels of inflammatory and oxidative stress markers between stroke cases and control subjects. It has evaluated which antioxidant could be associated with those markers among cases and controls. They found that plasma levels of 8-iso-PGF2α were significantly higher among stroke patients compared to controls. Also, it was observed an inverse correlation between 8-iso-PGF2α levels and plasma vitamin C concentration and a positive association between this marker and hsCRP concentrations, both among stroke patients (Sanchez-Moreno et al. 2004).

Heart Failure

Increased aldosterone levels have also been associated with heart failure pathophysiology and adverse clinical outcomes (Swedberg et al. 1990). Indeed, mineralocorticoid receptor antagonism with spironolactone use reduces morbidity and mortality of subjects with severe heart failure compared to placebo group (Pitt et al. 1999).

Oxidative stress may affect interstitial matrix of myocardial collagen (Siwik and Colucci 2004) contributing to cardiac remodeling. In this context, a study has shown a relationship of aldosterone and plasma 8-iso-PGF2α adjusted for confounders in patients with chronic, stable heart failure and left ventricular ejection fraction less than 0.40 (Matsumori et al. 2006). Also, another study have revealed that this marker of oxidative stress was increased in the pericardial fluid – independently of potential confounders – in symptomatic compared with asymptomatic heart failure subjects and gradually increased with the functional severity of heart failure assessed using the New York Heart Association (NYHA) classification. These findings suggest that 8-iso-PGF2α may be a potential marker able to indicate the progression from asymptomatic to symptomatic heart failure and the progressive impairment of cardiac functional capacity (Mallat et al. 1998).

Subjects with decompensated congestive heart failure also have marked increased oxidative stress. A short-term inotropic treatment (milrinone or dobutamine) significantly decreases plasma levels of the 8-iso-PGF2α oxidative marker, possibly for two reasons: (i) a direct action of the treatment or (ii) the improvement on cardiac function secondary to the treatment (White et al. 2006). Nevertheless, this finding has shown to be of potential clinical relevance since higher levels of oxidation biomarker are related to adverse effects on cardiac structure and function and, consequently, to poor prognosis in subjects with congestive heart failure (Sorescu and Griendling 2002; Castro et al. 2003).

Analytical Methods for Measurement of 8-Isoprostane

The measurement of isoprostanes in plasma or urine has emerged as the best option to have a marker of endogenous oxidative stress (Fam and Morrow 2003; Halliwell and Whiteman 2004). Indeed, 8-iso-PGF2α represents a major component of the "total" F2-isoprostane class measured in plasma or urine, which contributes to ease and reliable measurement. Various approaches are available for the measurement of F2-isoprostanes, such as gas chromatography-mass spectrometry (GC-MS) and immunoassays (Schwedhelm and Boger 2003).

Mass spectrometer is a more sensitive and specific method to quantify the levels of 8-iso-PGF2α (Morrow and Roberts 1999); however, this equipment is relatively confined to specialized laboratories conducting cutting-edge research. Thus, the application of this methodology to clinical investigation is still restricted requiring high-cost structure and a staff with analytical expertise to operate the quantitative analysis (Patrono and FitzGerald 1997; Mori et al. 1999; Chu et al. 2009). An alternative approach is to use an immunoassay. In this case, immunoassay results using an antibody that has already been described for 8-iso-PGF2α correlate reasonably with GC-MS measurement in urine. On the other hand, as limitation to this method, cross-reactivity with other prostaglandins and isoprostane metabolites – wherein few metabolites have been synthesized or identified in order to check for cross-reactivity – may occur in the plasma measurements. Although there is no

consensus to the best methodology for the 8-iso-PGF2α measurement, the chromatography should be considered as a more reliable approach compared to immunoassays (Morrow and Roberts 1994).

It is important to point out that since oxidation in the time of samples processing may occur, the protocol for collection and storage of samples must be carefully examined. A strategy to avoid this concern may be the inclusion of the phenolic antioxidant butylated hydroxytoluene (BHT) – final concentration of 5 mmol/L – to the samples in order to prevent further oxidation and have biased values. In addition, unless assayed immediately, samples should be frozen at −80 °C to avoid new sample oxidation (Morrow et al. 1990a).

Potential Applications to Prognosis, Other Diseases, or Conditions

Although there is a high recognition of the importance of oxidative stress in cardiovascular and non-cardiovascular diseases, the measurements of markers of oxidative damage or antioxidant status are still confined to research setting and have not yet been entered in the clinical screening. In part, this is believed to be a consequence of limitation recognition of the great majority of biochemical markers.

The development of isoprostane analysis represents a considerable advance compared to previously available tests, but further evidences are required to state if isoprostane measurement will be able to enter clinical use. In this concern, there is a need for prospective studies showing that increased plasma or urinary concentrations of isoprostanes predict hard cardiovascular or non-cardiovascular outcomes. If isoprostane analysis can be shown to provide additional prognostic information compared with other biomarkers and significantly reveal improvement in risk assessment, then clinical use will be more well advised.

Moreover, the standardization of analytical method for measurement of 8-iso-PGF2α is needed. Although immunoassays have been considered the most convenient for clinical laboratories, currently chromatographic techniques have been suggested as a superior method. Finally, there is also a need for a better understanding of isoprostane physiology, trying to include in studies its biological variability and the influence of environmental factors and disease (Young 2005).

The great majority of researchers have focused their works on studying association between oxidative stress and pathophysiology of cardiovascular disease, but non-cardiovascular conditions – such as pulmonary and neurological diseases as well as cancer and liver affections – have also acquired prominence in this concern.

Asthma and Pulmonary Diseases

Asthma, besides being characterized as an inflammatory response, is also known by the generation of a series of ROS that could affect cell structure and its

functionality (MacNee 2001), also due to its vasoconstrictor properties. Plasma 8-iso-PGF2α levels were found to be elevated in asthmatic subjects compared to age- and gender-matched healthy controls. A study that found this association showed that the biomarker levels were associated with clinical asthma severity and inhaled corticosteroid use in asthmatic patients. This last indicates that indices of oxidative stress may persist despite steroid use in these patients, which also may reflect that the treatment had not effectively controlled their asthma (Wood et al. 2000). On the other hand, a study including young adults in the general population found a lack of association of the 8-iso-PGF2α levels and the symptoms of asthma and some respiratory outcomes such as forced expiratory volume in the bronchial hyperresponsiveness to increasing doses of methacholine. These negative findings were explained by the fact that majority of individuals may have mild manifestations of the disease and are not likely exposed to an acute oxidative stress revealing a "normal range" of oxidative status (Garcia-Larsen et al. 2009).

Obstructive Sleep Apnea

Obstructive sleep apnea syndrome (OSAS) is known as the repetitive nature of partial or complete collapse of the upper airway. Indeed, oxidative stress in patients with sleep apnea has also been investigated as an important pathophysiological mechanism that links OSAS with endothelial injury and increased risk for cardiovascular outcomes such as atherosclerosis. It was encountered that intermittent hypoxia and cycles of hypoxia/reoxygenation may lead to increased vascular release of ROS (Mugge 1998).

Positive associations were found beyond plasma levels when assessing local oxidative stress in exhaled breath condensate of patients with OSAS (Carpagnano et al. 2002, 2003). Higher levels of 8-iso-PGF2α were seen in the morning exhaled condensate and plasma of OSAS patients compared to healthy obese subjects, and a reduction of its levels was seen after continuous positive airway pressure (CPAP) therapy. Moreover, the morning exhaled 8-iso-PGF2α levels correlated positively with the apnea-hypopnea index and neck circumference, suggesting that the 8-iso-PGF2α measurement in the exhaled breath condensate may help to identify patients with a higher risk of developing cardiovascular diseases (Carpagnano et al. 2003).

On the other hand, an observational prospective study including an OSAS population free of comorbidities did not find significant difference in the plasma 8-iso-PGF2α levels – neither in other biomarkers that reflect pathways of increased oxidative damage – between severe apnea patients and control subjects (Ntalapascha et al. 2013). In addition, neither untreated moderate-severe OSAS nor OSAS patients treated with CPAP nor normal sleep subjects affected the levels of 8-iso-PGF2α.

Although the findings mentioned above were negative, it should be noted that studied populations in some works designed to evaluate oxidative damage in OSAS

were highly diversified, which involved several comorbidities such as diabetes, hypertension, and mainly obesity; thus, the evidences linking OSAS with oxidative stress remain controversial.

Alzheimer Disease

Regarding reactive oxidative species-induced damage during aging and related diseases (Rao and Balachandran 2002), a study has evaluated the aging process with respect to the biomarker of in vivo lipid peroxidation, i.e., plasma 8-iso-PGF2α levels. The researcher group has investigated this marker in healthy subjects and in patients with Alzheimer, an age-related neurodegenerative common disease, in which oxidative stress is thought to be involved. The results showed that plasma concentrations of 8-iso-PGF2α were not modified with increasing age (Feillet-Coudray et al. 1999). On the other hand, another study observed higher levels of this biomarker in urine samples as a result of aging process. However, the study has been criticized for including few patients over age 60 years and thus achieving inconclusive findings (Wang et al. 1995).

Cancer

Oxidative stress plays a key role in carcinogenesis, and increased levels of ROS have been attributed to mutation and damage of normal tissues which facilitates susceptibility of tumor growth (Trush and Kensler 1991). A case-control study matched by age has compared patients with breast cancer at initial diagnosis to healthy subjects to evaluate the circulating oxidative stress-related markers such as 8-iso-PGF2α. The plasma concentration of this biomarker was not statistically different between the two groups, but another compound (the 8-hydroxy-20-deoxyguanosine, 8-OHdG) – also considered a reliable marker of oxidative stress level – and inflammatory markers IL-1β, and IL-6 were higher in cases compared to controls (Yeon et al. 2011). Furthermore, in contrast to the study hypothesis, elevated urinary 8-iso-PGF2α levels were significantly inversely associated with all-cause mortality in patients after primary breast cancer treatment and adjustment for potential confounding factors (Nechuta et al. 2014). This finding has suggested that lipid peroxidation may have a potential protective function in breast cancer etiology, although further elucidation is required (Gago-Dominguez et al. 2005). Overall, the lipid peroxidation has been investigated in cancer, but it seems that studies with plasma 8-iso-PGF2α levels remain limited.

Hepatitis

Lipid peroxidation and oxidative stress have also been studied in the pathogenesis of chronic liver diseases. Plasma 8-iso-PGF2α levels were evaluated in patients with

nonalcoholic fatty liver disease (NAFLD), with chronic hepatitis C and cured counterparts, and with hepatitis C virus-positive hepatocellular carcinoma and healthy volunteers. The biomarker levels were significantly higher in patients with chronic hepatitis C than in cured ones and normal controls. Moreover, 8-iso-PGF2α was also significantly higher in patients with NAFLD than in healthy subjects, which indicate that increased oxidative stress derived by lipid peroxidation is involved in the pathogenesis of NAFLD and chronic hepatitis C (Konishi et al. 2006).

Conclusion

The wide participation of 8-iso-PGF2α levels in pathophysiological mechanisms of cardiovascular disease – also in minor proportion, but not in minor importance, in non-cardiovascular diseases – may be of importance in the rational development of antioxidant drugs in humans. Indeed, the therapeutic potential of antioxidant treatment in cardiovascular disease, especially in atherosclerosis, is something to be pursued. In this concern, elevated levels of 8-epi-PGF2α have also been associated with the onset of different risk factors linked to atherosclerosis, supporting the hypothesis that the evaluation of oxidative stress compounds may represent additional prognostic predictors in such events, as well as targets for the development of novel strategies of pharmacological therapy.

However, it is important to note that the association observed in almost all studies does not imply a causal relationship. Since until now there are no clinical studies designed to test isoprostanes as a prognostic marker with hard end points such as mortality or morbidity, studies based on a narrowed population to avoid confounding factors are of great clinical interest. Therefore, it has been shown that the clinical measurement of 8-epi-PGF2α as a prognosis marker remains to be established, which in turn could be included in the future as a surrogate end point in clinical trials.

Summary Points

- Oxidative stress is a biological condition in which there is an imbalance in production of reactive oxygen species and the capacity of its damage repair by the biological system. The condition of increased oxidative stress is nowadays continuously recognized as a contributing factor to several chronic degenerative pathological conditions.
- Isoprostanes – a class of substances used as potential markers of oxidative stress in vivo – are associated with non-cardiovascular and mostly with cardiovascular diseases. This chapter focuses on its mainly studied part, the 8-isoprostane.
- Atherosclerosis is the most studied condition regarding oxidative stress. Atherosclerotic development, intensity of obstructive coronary artery disease, and acute coronary events have been consistently associated with oxidative stress.

8-Isoprostane is linked to these events through lipid peroxidation culminating in thrombotic complications.

- Hypertension is long known to be linked to oxidative stress. The increase in its biomarkers is shown to be associated with blood pressure (BP) altering through angiotensin II. Hence, the higher the endothelial dysfunction, the higher the isoprostane levels and enhanced BP.
- Although a great amount of observational studies suggest the link between these pathological conditions and 8-iso-PGF2α, no causal relationship can be inferred. However, this marker is already consolidated in these conditions as a marker of oxidative stress, and the relation to worse outcome is continuously growing.

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Irisin Concentrations as a Myocardial Biomarker

22

Suna Aydin and Suleyman Aydin

Contents

Abstract

Mortality and morbidity from acute myocardial infarction (AMI), in which cardiomyocytes are damaged and destroyed, are increasing daily worldwide. Early intervention may decrease morbidity and potentially mortality in AMI. Currently troponins are seen as superior to all other biomarkers, but they have a major limitation since they do not become elevated during the initial hours of AMI. Therefore it is important to find a better biomarker for early diagnosis (within the initial hours). In this sense, a new thermogenic uncoupling protein,

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_3

irisin, may be a useful marker to diagnose and make some prognosis regarding the long-term clinical outcome that might assist clinicians. Irisin is ubiquitously expressed in kidneys, liver, nerve sheath, skin, adipose tissue, and is abundantly synthesized in cardiac muscle. Serum and saliva concentrations also decrease with AMI. This chapter provides a brief description of AMI and current available tools for its diagosis, and some information about this candidate molecule irisin, including its concentration in human diseases. There is also an overview of isoproterenol (ISO)-induced myocardial infarction (MI) and irisin concentration in an animal model and how it changes in the serum and saliva of patients hospitalized after diagnosis of AMI. Finally, we discuss what should be known about the analysis of irisin in biological fluids. In summary, current data indicate that serum irisin measurement is probably a clinically effective test that offers some prognostic information to clinicians.

Keywords
Myocardial infarction • Irisin • Biomarker • Heart attack

Abbreviations

ACC	American College of Cardiology
ACS	Acute coronary syndromes
AMI	Acute myocardial infarction
CK	Creatine phosphokinase
CKD	Chronic kidney disease
ECG	Electrocardiogram
ELISA	Enzyme-linked immunosorbent assay
ESC	European Society of Cardiology
FNDC5	Fibronectin domain-containing protein 5
GDM	Gestational diabetes mellitus
GFR	Glomerular filtration rate
ISO	Isoproterenol
MACE	Major adverse cardiovascular events
MetS	Metabolic syndrome
MI	Myocardial infarction
NAFLD	Non-alcoholic fatty liver disease
PCOS	Polycystic ovary syndrome
T2DM- CKD	Type 2 Diabetes Mellitus with Chronic Kidney Disease
T2DM	Type 2 Diabetes Mellitus
UCP1	Uncoupling protein1
WHO	World health organization

Definitions

Acute coronary syndrome(s) Sudden obstruction of blood flow to the heart entailing myocardial infarction, with unstable angina (intense pain).

Acute myocardial infarction Restriction of oxygen supply to the cardiac tissues, leading to death of cardiomyocytes, commonly referred to as a heart attack.

Biomarker Indicator of the severity of disease, usually raised compared with that normally seen in a healthy state.

Glycoprotein hormone Protein hormone conjugated with a carbohydrate moiety.

Irisin Myokine protein needed for fat metabolism which can control energy production under certain conditions.

Major adverse cardiovascular events Adverse cardiovascular events, including acute myocardial infarction, ischemic stroke, coronary occlusion and death.

Uncoupling protein Mitochondrial inner membrane protein that dissipates proton gradients.

Introduction

AMI is the major cause of morbidity and mortality worldwide with a steady increase in incidence (Ahmed et al. 2014). Approximately 15–20 million patients annually seek care in emergency, cardiology, and cardiovascular surgery departments in Europe and the USA with acute chest pain or other symptoms suggestive of AMI, but only ∼10 % are subsequently confirmed to have infarction (Lewandrowski et al. 2002). A more rapid identification of these patients would substantially reduce overcrowding in these departments, which many have shown is a major problem associated with increased morbidity and even mortality. The World Health Organization (WHO) has recommended noting two of three characteristic criteria (Mendis et al. 2011): (1) clinical history of chest discomfort of >30 min duration, (2) typical rise and fall in cardiac markers [currently creatine phosphokinase (CK) and its isoenzyme CK-MB] indicating myocardial muscle tissue injury), and (3) New Q waves on ECG (unequivocal ECG changes) to differentiate AMI from healthy persons at any time after onset of symptoms. The European Society of Cardiology (ESC)/American College of Cardiology (ACC) criteria are typical rise and fall of cardiac markers accompanied by one of the following; new Q waves, ischemic symptoms, ischemic electrocardiogram (ECG) changes, and coronary intervention (Aguero et al. 2014; Alpert et al. 2000: Fihn et al. 2012; Leonardi et al. 2012). Measurement of cardiac markers in the blood has been used for diagnosis of AMI for nearly 60 years (Porela et al. 2000; Tucker et al. 1997). The chief markers and their specificity and sensitivity used to diagnose AMI are given in Table 1 (Zimmerman et al. 1999).

The ideal biological marker should reliably have a sensitivity of ≥90 % and exclude others by its specificity being >90 % (Zimmerman et al. 1999). It needs to be found in the blood or other body fluids, tissues, and organs in quantifiable amounts in pathological conditions, but not under normal conditions. However, as

Table 1 Sensitivity and specificity of commonly used biomarkers and candidate biomarker irisin for the evaluation of AMI, based on time after injury (None of the currently used biomarkers as 100 % sensitivity and specificity, and that of irisin in AMI diagnosis is currently unknown)

	Times (h)			
	6		14	
Parameters	Sensitivity	Specificity	Sensitivity	Specificity
TnI (1.5 ng/mL)	57.5	94.3	90.6	92.2
TnT (0.1 ng/mL)	61.7	96.7	84.9	96.1
MB Activity (9 IU/L)	74.5	97.5	98.1	96.1
MB Mass (7 ng/mL)	66	100	90.5	98.9
MB Subforms (1.6 ratio)	91.5	89	90.6	90
Myoglobulin (85 ng/mL)	78.7	89.4	62.3	88.3
Irisin	?	?	?	?

indicated above, currently no biomarker fulfills these criteria. The risk of death is highest within the first few hours after AMI onset. Delay in intervention therefore increases morbidity and potentially mortality. Many investigations have recently been introduced, in which several serum proteins have been assessed to determine their sensitivity and specificity for the detection of myocardial damage to avoid delay in "ruling out" AMI; these include heart-type fatty acid binding protein (Cappellini et al. 2013), adropin (Aydin et al. 2014b; Yu et al. 2014), copeptin (in combination with cardiac troponin) (Jayasinghe et al. 2014), and the newly discovered myokine protein irisin (Aydin et al. 2014a; Emanuele et al. 2014; Kuloglu et al. 2014).

This chapter first deals briefly with the discovery of irisin, its tissue distribution, and its biochemical and physiological effects. The reader will find more detailed information in the reports and reviews published in the literature (Sanchis-Gomar et al. 2014; Aydin 2014). Second, this chapter will deal with the clinical application of irisin in the early diagnosis of AMI. It is hoped that the continuing studies of this protein will improve the early diagnose of AMI and guide intervention in positive cases that should decrease morbidity and potentially mortality.

Irisin

Boström et al. discovered irisin in 2012, a 112 amino acid peptide (Fig. 1) found in rat and human skeletal muscle after exercise (Boström et al. 2012). It acts on white adipose cells in culture and, in vivo, stimulates the expression of uncoupling protein 1 (UCP1) and browning of white fat cells in mice (Boström et al. 2012). Irisin is named after the Greek personification of the rainbow as Iris (the name of a flower or girl) who was a messenger for the Olympian Gods (Boström et al. 2012). After pioneering study, it was found that irisin is almost ubiquitously expressed in bodily tissues (Fig. 1), notably the liver, kidneys, lungs, adipose tissue, exocrine glands, spleen, salivary glands (review, Aydin 2014; Aydin et al. 2014c), and cardiac tissues (Fig. 2).

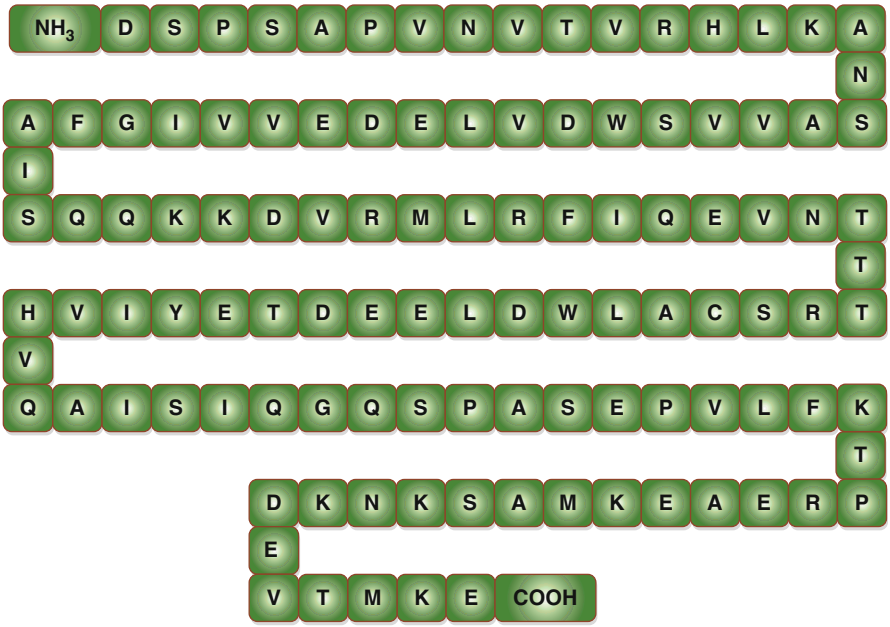

Fig. 1 Amino acid sequencing of rat, mouse and human irisin. The homologous peptides from rats, mice and humans have a similarity of 100 %

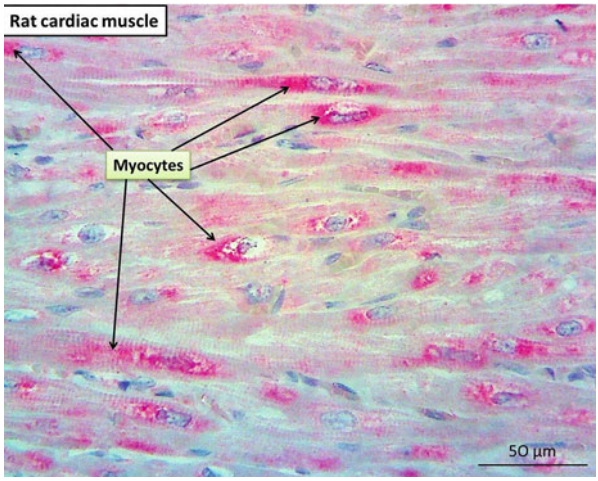

Fig. 2 Irisin expression in the myoctes (*red color*) was detected immunohistochemically with Avidin-Biotin-peroxidase Complex (*ABC*). Specific irisin antibody (Phoenix Pharmaceuticals, Inc., CA, USA (cat no: H-067-17)

This hormone is released upon cleavage of the plasma membrane protein, fibronectin type III domain-containing protein 5 (FNDC5) (Boström et al. 2012). The FNDC5 gene is located on chromosome 1p35.1 encoding a 203 amino acid protein (Staiger et al. 2013). The amino acid sequences of mature irisin are well conserved, with human and mouse proteins being 100 % identical. An irisin receptor has still to

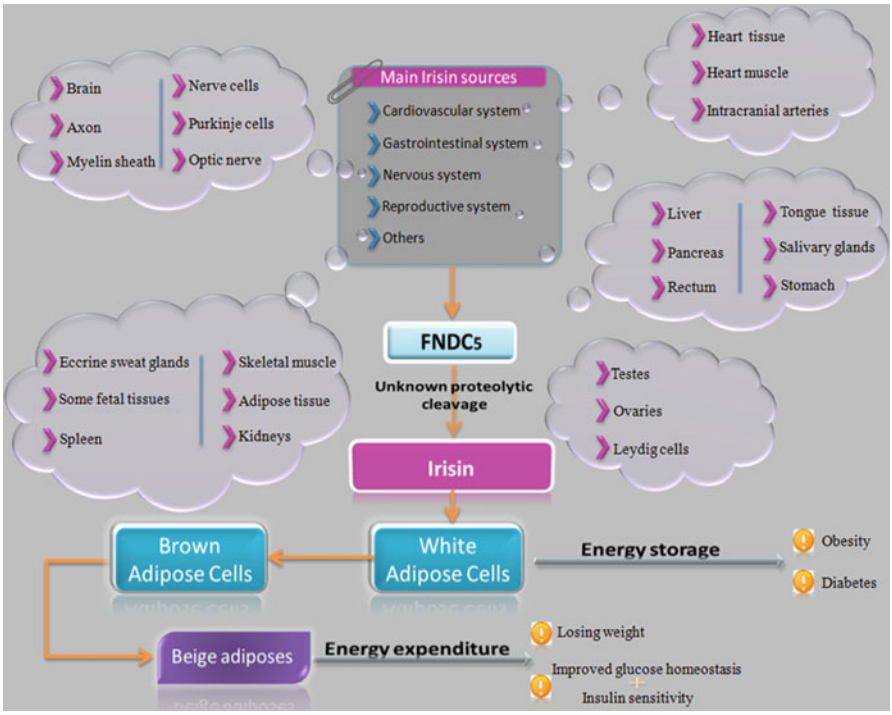

Fig. 3 Main irisin sources in biological systems and its known biochemical and physiological functions. *FNDC5* fibronectin type III domain-containing protein 5

be identified (Fig. 3). The highest basal levels of FNDC5 expression are seen in brain and heart, with low basal levels in liver, lung, skeletal muscle, and testis (Ferrer-Martínez et al. 2002). After FNDC5 cleavage by an unknown protease (Fig. 3), irisin is released from muscle and other cells, such as adipose tissue and liver cell, enters the circulation, and becomes detectable in murine and human blood and also in human saliva (review, Aydin 2014).

Physiological Function of İrisin

İrisin has many physiological functions; one of its most important is to stimulate the expression of uncoupling protein 1 (UCP1) and the browning of the white fat cells in mice. In this way, irisin promotes brown adipocyte recruitment in white fat and improves systemic metabolism by increasing energy expenditure (leading to weight reduction) and improves the parameters of glucose metabolism that can affect obesity and increase muscle strength (Boström et al. 2012). The pancreas also produces irisin, but its function here is unknown (Aydin 2014). It was assumed that pancreatic irisin could be a regulator of insulin secretion because its inhibition enhances insulin release to meet the increased demand in diabetic subjects, in whom irisin was decreased. İrisin might be master hormone to decide whether energy is

released as heat or stored as ATP. If it is the former, its synthesis is upregulated in tissues and more is released into the circulation, thereby converting more white adipose tissue to brown adipose tissue, with the stimulation of uncoupling protein 1 (UCP1) expression leading to energy being released as heat, or vice versa (Fig. 3).

Alteration of Irisin Concentration in Human Diseases

Most studies have focused on relationship between exercise and irisin concentration in human and animal subjects. However, controversial results and conclusions have been reported, which will not be discussed here, although details are available in two review articles. We only focus below on alterations in irisin levels in human dieases, especially AMI.

The best-known factor regulating the rate of irisin secretion into serum and skeletal muscle tissues is exercise. The relationship between muscle mass and circulating irisin is controversial after different types of exercise, which will not be covered here since details can be found in the review articles mentioned above (Aydin 2014). Therefore, we will focus on only alteration of irisin concentration in some human diseases, those on interest being summarized in Table 2, in which some of the data also is controversial for certain disorders.

Circulating irisin is significantly lower in long-term type 2 diabetic patients compared with nondiabetic controls (Moreno-Navarrete et al. 2013; Alis et al. 2014; Choi et al. 2013; Liu et al. 2013; Xiang et al. 2014; Kurdiova et al. 2014), and the lower serum irisin is also found in type 2 diabetic patients compared with nondiabetic controls (Moreno-Navarrete et al. 2013), which includes in new-onset shown by Choi et al. 2013, as also in undefined type 2 diabetic patients by Moreno-Navarrete et al. 2013, respectively. Liu et al. also reported that diabetic patients with CKD have a lower irisin concentration than control subjects (Liu et al. 2014). In contrast, the levels of irisin fasting were significantly elevated in type 2 diabetes patients when compared to matched controls. Type 2 diabetes shares the same pathology as gestational diabetes mellitus regarding insulin resistance and elevated glucose levels. It is generally indicated that serum irisin levels are significantly decreased in subjects with gestational diabetes mellitus (GDM) than control groups. Serum levels increase markedly in pregnant women but are significantly lower in patients with GDM. Conversely, one study found that maternal serum irisin levels of patients with GDM are significantly higher than non-GDM controls (Ebert et al. 2014b). These results may imply that irisin has a protective role in the pathology of insulin resistance and related conditions, such as metabolic syndrome and type 2 diabetes.

Type 2 diabetes, GDM, obesity, MetS, and PCOS share the same pathology of insulin resistance. However, there is no consensus regarding serum irisin levels; some studies show that serum levels are significantly lower in subjects with MetS and obesity, whereas others found them significantly higher (Table 2). Serum irisin levels were also significantly raised in subjects with PCOS. There is no consensus on the relationship of irisin concentration and BMI, some reports showing them to be

Table 2 Irisin concentrations due to diseases in humans

Disease	Samples	Method	Results	Comments	References
T2DM	Serum/ plasma	ELISA	↓	Decreased irisin level might be linked with these diseases	Moreno-Navarrete et al. (2013), Alis et al. (2014), Choi et al. (2013), Liu et al. (2013), Xiang et al. (2014), Kurdiova et al. (2014).
T2DM-CKD	Serum	ELISA	↓		Liu et al. (2014)
CKD	Serum	ELISA	↓		Ebert et al. (2014a)
GDM	Cord/ maternal serum/ milk	ELISA	↓		Aydin et al. (2013a), Ebert et al. (2014b), Kuzmicki et al. (2014), Yuksel et al. (2014)
MI	Serum/ plasma	ELISA	↓		Aydin et al. (2014a), Kuloglu et al. (2014), Emanuele et al. (2014)
NAFD	Serum	ELISA	↓		Polyzos et al. (2014)
MetS	Serum	ELISA	↓↑	No consensus	Park et al. (2013), De la Iglesia et al. (2014), Yan et al. (2014)
Obesity	Plasma/ serum/ saliva	ELISA	↓↑		Aydin et al. (2013b), Moreno-Navarrete et al. (2013), Polyzos et al. (2014), Stengel et al. (2013)
ACS	SERUM	ELISA	—	No association	Aronis et al. (2013)
MACE	Serum	ELISA	↑	Increased irisin level might be linked with these diseases	Aronis et al. (2013)
PCOS	Serum	ELISA	↑		Chang et al. (2014)

T2DM type 2 diabetes mellitus, *T2DM- CKD* type 2 diabetes mellitus with chronic kidney disease, *CKD* chronic kidney disease, *GDM* gestational diabetes mellitus, *MI* myocardial infarction, *NAFLD* non-alcoholic fatty liver disease, *MetS* metabolic syndrome, *ACS* acute coronary syndromes, *MACE* major adverse cardiovascular events, *PCOS* polycystic ovary syndrome, *ELISA* enzyme-linked immunosorbent assay

negatively correlated, others indicating a positive correlation, and another group who found no correlation. Future studies need to clarify the association of serum irisin with different adiposity indicators.

Serum irisin concentration decreases markedly in T2DM with renal insufficiency, leading to a glomerular filtration rate (eGFR) of $\geq$60 ml/min/1.73 m^2 compared with subjects where the value is <60 ml/min/1.73 m^2. Serum irisin concentrations decrease with increasing chronic kidney disease (CKD) stage in subjects, when adjusted for age, gender, and BMI, being independently and positively predicted by renal function and insulin resistance (Ebert et al. 2014a). Irisin concentration is also associated with facets of metabolic syndrome, including diastolic blood pressure, markers of impaired glucose tolerance, and dyslipidemia. Serum irisin is lower in obese patients (Aydin et al. 2013b; Moreno-Navarrete et al. 2013; Polyzos et al. 2014; Stengel et al. 2013), those with nonalcoholic fatty liver (NAFL), and

in cases of nonalcoholic steatohepatitis (NASH) compared with their lean counterpart controls; however, they are similar among patients with NAFL, NASH, and their obese controls (Polyzos et al. 2014). Serum irisin tends to be higher in patients with than without portal inflammation, and is independently associated with the latter. Irisin levels do not predict the development of acute coronary syndromes (ACSs) in healthy individuals, but elevation is associated with the development of major adverse cardiovascular events (MACE) in patients with established coronary artery disease after percutaneous coronary intervention (Aronis et al. 2013).

Serum irisin assayed by ELISA showed significant decrease in patients with MI compared to matched controls (Aydin et al. 2014a; Kuloglu et al. 2014; Emanuele et al. 2014). The levels and their MI relationships has been the main purpose of this chapter, therefore we give more details in the coming sections. Overall, even though there were contradictory results, all studies indicate that irisin may have both peripheral and central roles in human metabolic disease. However, changes of irisin with diseases needs further investigation in future experiments.

Association of Irisin with AMI

The myocardium receives its blood supply through the right and left coronary arteries, not the blood it constantly pumps through its chambers. If blood flow in the heart tissues is suddenly reduced or stopped, cardiac muscle undergoes necrosis, resulting in a heart attack – AMI (Ahmed et al. 2014). The myocardium can generate energy from fatty acids, glucose, lactate, pyruvate, amino acids, and ketone bodies. The energy within heart muscle is stored as either adenosine triphosphate (ATP) or kreatin phosphate (KP). Under a fluent and sufficient supply of oxygen and substrates, ADP and Pi formed by splitting are resynthesized to ATP; the energy necessary for these processes is provided by KP (Mallet and Sun 1999).

Preference from these substrates for energy generation depends on their concentration in both blood and cardiac muscle cells. If the oxygen supply is adequate, the dominant fuel is fatty acids that cover 50–70 % of the total energy demands of the myocardium, with glucose covering the other 30 %. AMI is characterized by an extraordinarily high energy demand and oxygen deficiency, with 30 min of ischemia reducing myocardial ATP by 50 %, thereby decreasing cardiac muscle function (Turdi et al. 2011). The store of KP in the cardiac muscle is too small and can only retain a small amount of ATP for short time. Energy homeostasis would be tightly controlled by irisin levels - a signal for energy availability during AMI to avoid ischemia in the heart.

In this regard, irisin expression examined immunohistochemically in rat heart, skeletal muscle, kidney and liver in isoproterenol (ISO)-induced AMI, and serum irisin concentration by ELISA have now been analyzed by Kuloglu et al., who reported a gradual decrease in serum irisin from 1 to 24 h rats compared with the controls, the minimum being at 2 h, increasing again after 4 h, but this increment was still lower than normal even at 24 h compared with control irisin concentration (Kuloglu et al. 2014). They also showed that irisin expression in the cardiac muscle cells, glomerular, peritubular renal cortical interstitial cells, hepatocytes and liver

sinusoidal cells, perimycium, endomycium, and nuclei of skeletal muscle tissues decreased 1–4 h after AMI. Furthermore, irisin was raised near myocardial connective tissue at all time points, with production in skeletal muscle, liver, and kidney recovering after 6 h after AMI (Kuloglu et al. 2014).

The same research team had examined the time-dependent change in irisin levels after AMI in another study and found that it decreased in a time-dependent manner in saliva and serum from first hour up to 48 h compared with the control group, whereas troponin-I, CK, and CK-MB AMI group gradually increased for up to 12 h. They also showed that irisin expression in the three major paired salivary glands (submandibular, sublingual, and parotid) produce and release irisin into saliva (Aydin et al. 2014a). Others have collectively investigated whether there is an association between serum irisin levels, precocious myocardial infarction, and exceptional longevity. They found that healthy centenarians had increased serum irisin levels, whereas young patients with myocardial infarction had lower levels (Emanuele et al. 2014).

One important question from published results rose, that of why irisin decreases with AMI rather than increases. Because irisin is found in myocardium of the heart (Fig. 2), skeletal muscle, and some other tissues, it should be released rapidly into blood from infarcted myocardium, like troponin and CK-MB, which are also synthesized in the myocardium. It was argued that instead of a decline in irisin during AMI, sudden release occurred into the circulation, which could be regarded as the basic metabolic problem; the consequences include changes that predictably worsen ischemic damage due to contributing to a drop in total ATP. If tissues did not react in this way, more irisin would lead to greater energy depletion and more heat production. Thus heart tissues starved of energy would become necrotic more quickly; thus a progressive decrease in serum and tissue irisin levels is a means of protecting myocardial cells by saving the extra energy that would otherwise have to be given to ischemic mycardiocytes, achieved by inhibiting ATP loss. They also assumed that, if irisin level is not decreased in MI, myocardial and other cells would experience more damage due to irisin-dependent loss of ATP (Aydin et al. 2014c). A strict relationship exists between oxygen consumption and cardiac work that occurs at steady global cellular ATP and phosphocreatine (PCr) concentrations. By blocking *de novo* irisin production, they assume that heart tissues will be saved and protected to some extent from damage. Here we also ague that İrisin is formed after the membrane-anchored protein fibronectin type III domain containing protein 5 (FNDC5) is cleaved to generate the secreted protein. It is this characteristic that might affect its release under different pathophysiological conditions (Boström et al. 2012).

Furthermore, impaired cardiac function is detrimental to the liver and the kidney (cardiorenal interaction). As indicated in Fig. 3, these tissues are good sources of irisin. Detrimental effects of AMI on liver and the kidney tissues might lower irisin synthesis due to damage or in order to save ATP; thus, irisin synthesis might be downregulated in liver and kidney tissues by the mechanism suggested above. Overall, besides cardiac troponin and CK-MB, irisin adds novel diagnostic information regarding AMI patients.

Can Irisin Be a Biomarker in the Acute Myocardial İnfarction?

Biological markers are substances found in the blood or other body tissues and fluids in quantifiable amounts, whose detection indicates a particular disease state, but are not detectable or are in much smaller amounts in the normal state of the body. The amount of these substances should alter as a physiological response to therapeutic intervention (Tainsky 2009). In order for a biological molecule to be accepted as a biomarker, it should show an obvious change in amount or concentration that is indicative of only a particular disease. An ideal biomarker should be easily assessed in tissues or body fluids in the diseased state; its concentration or activity should be measurable in an inexpensive, reliable, rapid, and repeatable test and should not vary widely in the general population (Aydin 2013) Specificity and sensitivity comparisons should show at a particular point on the individual receiving operating curves (ROC) where likelihood ratios are equivalent and clinically meaningful. Thus, this can help clinicians in the diagnosis and follow-up of patients. Does irisin meet these criteria?

The relevant data are currently quite limited for both humans and experimental animals. Evidence does show that irisin decreases in the cardiac injury, thus can be of value in the diagnosis of AMI. Especially in humans, irisin significantly decreases after 1–48 h from the onset of the chest pain and returns more or less to baseline after 72 h, making it of value in diagnosing AMI (Aydin et al. 2014a). However, it is not known whether decreased irisin is associated with adverse outcomes in many other clinical situations, including congestive heart failure, chronic kidney disease, acute pulmonary embolism, and sepsis. Furthermore, irisin is not specific to the cardiac muscle because it is also found in high concentrations in the liver, kidney, and other organs (Fig 3). Irisin is notably decreased with AMI, unlike serum creatine kinase (CK), CK-MB activity, CK-MB mass, LDH, AST, troponin I and T (gold standard), all of which have been historically been used as biomarkers of AMI. This properity of irisin will help to find it a place in clinical use for the diagnosis of AMI.

In general and before using irisin as a diagnostic of AMI, uncertainties and questions need answers, including its cutoff values, sensitivity and specificity, use of the ROC curve optimum value of irisin to compare against “gold standard cTnT, and cTnI” highly sensitive and specific for cardiac damage assay that differs in patient populations, prospective double-blind study with large populations that compare CK-MB mass, CK-MB subforms, and CK-MB activity. New rapid automated laboratory techniques are needed for irisin measurement beside ELISA techniques that might exclude AMI.

Analysis of Irisin in Biological Fluids

Irisin is usually measured by ELISA, but the quantification varies greatly between the kits. Thus, one cannot rely on reports of a serum or plasma concentration in a normal person that varies from 389 ng/mL (not within the range of most ELISA kits), or 257 ng/mL (USCN LifeScience), to 65–1000 ng/mL (Phoenix), or

Table 3 Suppliers of irisin kits

Methods	Catalog #	Company name	City	Country
ELISA/ EIA	EK-067-52	Phoenix Pharmaceuticals, Inc.	California (CA)	USA
	EK-067-29			
	EK-067-19			
	EK-067-17			
	EK-067-16			
	Aviscera Bioscience	Aviscera Biosciences	Santa Clara/CA	USA
	SEN576Hu (human)	Uscn Life Science Inc.	Wuhan	CHINA
	SEN576Mu (Mouse)			
	AG-45-0046EK-KI01	Adipogen International, Inc.	San Diego/CA	USA
	RAG018R	BioVendor	Brno	Czech Republic
	MBS706887	MyBiosource	San Diego/CA	USA
	CSB-EQ027943HU	Cusabio	Wuhan	P.R. China
RIA	RK-067-16 (Human, Mouse)	Phoenix Pharmaceuticals, Inc.	California	USA

ELISA enzyme-linked immunosorbent assay, *EIA* enzyme immunoassay, *RIA* radio-imnuno-assay

50–2157 ng/mL [too high; Aviscera Bioscience]. These differences probably come from the variety in the irisin epitopes being targeted for measurement by the manufacturing companies. New methods should be developed and standardized tests should be designed to measure accurately the amino acid sequencing of FNDC5/irisin. Plasma/serum and saliva irisin concentrations measured by ELISA kits gave controversial results, and conclusions have been reviewed and summarized by Aydin (2014) and Sanchis-Gomar et al. (2014). Major ELISA irisin kit suppliers are listed in Table 3.

When an accurate measurement of irisin concentration is needed, appropriate amounts of protease inhibitors should be put into biological sample tubes (such as for saliva, blood, or urine) or Eppendorf tubes before their collection of biological fluids, and into which supernatants from homogenized tissues might also be transferred. It is generally recommended to use 500 kallikrein inhibitor units (KIU) of aprotinin per 1 ml of biological samples. Proteases have been reported to be encoded in >700 human genomes, according to MEROPS data records (Rawlings et al. 2012). In the case of disease, the concentration of proteases changes in response to the particular disorder. If protease inhibitors are not used, irisin will be degraded by proteases, making the irisin concentration unexpectedly low.

Potential Applications to Prognosis, Other Diseases or Conditions

Irisin has recently been identified as an exercise-induced glycoprotein hormone secreted by numereous tissues, including adipose, cardiac, and skeletal muscle tissue in mice and humans. It is a cleaved version of FNDC5 (fibronectin domain-

containing [protein] 5) with unknown action. This protein has direct effects on "browning" of white fat that would lead to burning to produce excess calories (heat). In AMI, irisin secretion is associated with energy availability and expenditure, suggesting that it is involved in regulation of energy balance in deficiency states. Irisin measurement in human studies provides important clues to the pathogenesis of some diseases, since it characteristically declines with the progress of AMI in both humans and animal models.

The current tools available - patient history, new Q waves on ECG, typical rise in cardiac markers - are unable to rule in or out AMI in a substantial number of patients, which is especially true in the first few hours after infarction. Recently, several studies indicate that serum and saliva irisin measurement might help monitoring over a period of 1–48 h, and serial blood sampling to show a fall of irisin levels should be interpreted in the context of clinical and ECG findings, along with increased troponin and cardiac enzyme levels. Human irisin concentration studies in AMI cases have been the relatively small sample size. A larger, multicenter study might lead to generalization of a firm irisin assay for AMİ patient diagnosis. Furthermore, diagnostic accuracy should be quantified by the area under the receiver-operating-characteristic curve (ROC) in comparison with the gold-standard troponin assay, because areas under ROC curves for serum/plasma or saliva concentrations of this biomarker are not currently known relative to those of gold-standard tropin values in terms of their prognostic value.

Currently irisin assays are not consistent. A sensitive assay for irisin will be a step forward with respect to overall accuracy in the diagnosis for myocardial infarction, and its cost should be less or at least no more than the current gold standard tests. There are also no additional laboratory platform requirements. In summary, these concerns should be answered regarding the measurement of serum and saliva irisin concentration to make it a good and reliable biological marker identifying or excluding AMI before moving to full clinical application.

Summary Points

- AMI is one of the main causes of morbidity and mortality worldwide, its incidence increasing daily.
- Early detection might reduce morbidity and mortality.
- Currently the early biochemical gold-stardard biomarker diagnosis is troponin, but delays might "rule out" AMI as a diagnosis on this basis.
- Doctors need better biochemical markers to be sure of AMI. In this sense, ISO-induced AMI in animal models show that irisin production decreases in the liver, kidney, cardiac muscle tissue, and serum.
- Irisin also shows a similar characteristic decline during the progress of AMI in humans.
- However, there is insufficient evidence to date and not enough data to consider the value of irisin as a predictor in the diagnosis of AMI for it to be applied clinically.

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Part III

Specific Diseases and Conditions

New Role of Biomarkers in Atrial Fibrillation 23

Ana I. Rodríguez-Serrano, María A. Esteve-Pastor, Diana Hernández-Romero, Mariano Valdés, Vanessa Roldán, and Francisco Marín

Contents

Abstract

Atrial fibrillation (AF) confers a raised risk of stroke, thromboembolism, and death, and this risk of adverse events is increased by the coexistence of other cardiovascular risk factors. Despite being easy to use for decision-making concerning oral anticoagulant therapy in AF, different clinical risk scores used for stratification have shown modest capability in predicting thromboembolic

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_36

events, and biomarkers may improve our identification of "high-risk" patients. Biomarkers significantly improve risk stratification in addition to current clinical risk stratification models. These new findings may enable development of novel tools to improve clinical risk assessment in AF. This chapter will highlight novel associations of biomarkers and outcomes in AF as well as recent progress in the use of biomarkers for risk stratification, with focus on data from randomized prospective clinical trials and large community-based cohorts.

Keywords

Atrial fibrillation • Biomarkers • Stroke • Thromboembolism • Mortality • Bleeding

Abbreviations

AF	Atrial fibrillation
ARISTOTLE trial	Apixaban for the Prevention of Stroke in Subjects with Atrial Fibrillation trial
ATM	Antithrombin III
BNP	B-type natriuretic peptide
BTG	Beta-thromboglobulin
BTP	Beta-trace protein
CKD	Chronic kidney disease
CRP	C-reactive protein
DD	D-dimer
F1+2	Prothrombin fragment 1+2
FMD	Flow-mediated dilatation
GDF-15	Growth differentiation factor 15
GFR	Glomerular filtration rate
IL-6	Interleukin-6
INR	International normalized ratio
LV	Left ventricle
NT-proBNP	The inactive N-terminal fragment of B-type natriuretic peptide
OAC	Oral anticoagulation
PAI-1	Plasminogen activator inhibitor
PAP complexes	Plasmin–antiplasmin complexes
RE-LY trial	Randomized Evaluation of Long-Term Anticoagulant Therapy trial
sE-sel	Soluble E-selectin
SPAF III study	The third Stroke Prevention in Atrial Fibrillation study
sTM	Soluble thrombomodulin
TIA	Transient ischemic attack
TnI	Troponin I
TnT	Troponin T
tPA	Tissue plasminogen activator
vWF	von Willebrand factor

Key Facts of Stroke and Systemic Thromboembolism

- The pathways underlying thrombogenesis in AF are complex. Abnormal changes are consistent with a prothrombotic or hypercoagulable state in AF.
- Clinical scores such as $CHADS_2$ or CHA_2DS_2–VASc have only modest predictive value for predicting "high-risk" subjects and could benefit from inclusion of biomarkers related to prothrombotic changes.
- Several studies have reported troponins as an indicator of high risk of stroke or systemic embolism.
- In the larger ARISTOTLE and RE-LY substudies, an especially strong association between increased risk of ischemic stroke and rising NT-proBNP levels was observed.
- A recent study published by our group showed that adding CKD to the stroke risk scores did not independently improve the predictive value of current clinical scores.
- Rising cystatin C levels were independently associated with increased rates of stroke or systemic embolism.
- The addition of BTP improves the predictive value of a clinical risk score for the detection of thrombotic events.
- The recent trials describe a significant association between baseline D-dimer levels and the risk of stroke independent of established risk factors including CHA_2DS_2–VASc variables.
- Several studies have demonstrated an association between stroke and thromboembolism and endothelial damage biomarkers (vWF, sE-sel, sTM).
- A small study reported the association between IL-6 and a composite outcome of stroke and death.
- Platelet size, measured as mean platelet volume, has been associated with platelet reactivity and as an independent risk factor for future stroke.
- Adiponectin could exert a protective role against cardiovascular diseases.
- Although these markers are widely and effectively used in experimental research, their usage in everyday clinical practice remains limited.

Key Facts of Mortality

- Isolated increases of troponin I concentrations generally seemed to be associated with higher risk of cardiac death and myocardial infarction compared with isolated increases in troponin T.
- NT-proBNP was also predictive of all-cause mortality, suggesting that this biomarker may potentially be used to refine clinical risk stratification in anticoagulated patients with AF.
- The presence of impaired renal function was also associated consistently with the development of adverse cardiovascular events and mortality, even after adjusting for the $CHADS_2$ score.
- CRP and IL-6 seem to be independent markers for mortality in patients with AF.

- GDF-15 was shown to be an additive prognostic marker for death, even after adjusting for clinical variables, risk factors, and CHA_2DS_2–VASc score and other biomarkers.

Key Facts of Bleeding

- The causality is unknown, but elevated troponin I levels might contribute to the identification of a more fragile AF subpopulation more likely to bleed during anticoagulation.
- Higher baseline NT-proBNP concentration was strongly associated with each of the major clinical outcomes explored (like stroke or mortality), except major bleeding, even after adjustment for multivariable model.
- Renal dysfunction has been associated with an increased risk of bleeding. The incidence of major bleeding increased significantly with the impairment of renal function.
- Renal dysfunction may be associated with INR and worse the average percentage of the time in the optimal therapeutic INR (TTR).
- High levels of cystatin C, when added to the eGFR equation, were associated with increased HR rates of major bleeding in patients taking warfarin.
- BTP also improved the predictive value of the HAS-BLED score for major bleeding.
- A high plasma vWF level showed an additive effect on the HAS-BLED score, for an intermediate-risk category for bleeding (HAS-BLED score 1–2 points).
- DD levels at baseline, regardless OAC, were related to major bleeding.
- GDF-15 was shown to be an additive prognostic marker for major bleeding in patients with AF receiving.

Definitions

Atrial fibrillation An abnormal and irregular heart rhythm in which electrical signals are generated chaotically throughout the upper chambers (atria) of the heart.

Beta-thromboglobulin A platelet-specific protein that indicates platelet activation and is released from alpha-granules during platelet aggregation and subsequent thrombus formation.

Biomarker Any measurable indicator that are potentially useful along the whole spectrum of the disease process; research and development of new therapies; diagnosis, prognosis, and monitoring progression of a disease; or response to treatment.

B-type natriuretic peptide A neurohormone synthesized by myocytes, predominantly in the left ventricle, in response to increased wall tension such as volume or pressure overload.

CHA_2DS_2–VASc score A stroke risk score that assigns 1 point each for presence of congestive heart failure, hypertension, diabetes mellitus, vascular disease, age >65, and sex category (female gender) and 2 points to age >75 years and prior stroke/TIA. Patients with high $CHADS_2$ scores (>2) are at significant risk for stroke: 5.9 % annual risk with a score of 3 and up to 18.2 % annual stroke risk for patients with a score of 6.

Cystatin C A small protein, synthesized at a constant rate in all nucleated cells. It is freely filtered by the glomerulus and does not return to the blood flow.

HAS-BLED score A new bleeding risk score, ranges from 0 to 9 that assigns 1 point for the presence of each of the following: hypertension (uncontrolled systolic blood pressure >160 mmHg), abnormal renal and/or liver function, previous stroke, bleeding history or predisposition, labile international normalized ratios, elderly, and concomitant drugs and/or alcohol excess. With scores of ≥3 indicating high risk of bleeding, caution and regular review of the patient are recommended.

Plasma D-dimer A fibrin degradation product and is a marker of intravascular thrombogenesis and fibrin turnover.

TTR Percentage of the time of INR in the therapeutic range (2.0–3.0).

von Willebrand factor An established biomarker of endothelial damage/dysfunction. It is synthesized by vascular endothelial cells and promotes platelet adhesion and aggregation, leading to thrombus formation.

Introduction

Atrial fibrillation is the most common sustained cardiac arrhythmia which is associated with high risk of stroke, thromboembolism, and mortality (Wolf et al. 1991; Benjamin et al. 1998). In addition to these complications, many patients with AF have impaired cognitive function, impaired quality of life, and increased health care costs. The prevalence of AF increases with age and reaches 10 % in persons >80 years. The pathophysiology of AF is complex and multifactorial. The process involves a structural remodeling in which connective tissue deposition and fibrosis are the hallmarks (Fig. 1), as well as altered atrial electrophysiological properties facilitating the initiation and perpetuation of AF (Daoud et al. 1996; Frustaci et al. 1997). Besides, left ventricular dysfunction and elevated ventricular filling pressures contribute to atrial remodeling and may produce a substrate that predisposes for AF as well (Savelieva and Camm 2004). Many AF patients remain asymptomatic with an increase for fatal or disabling complications as first manifestation of this arrhythmia; consequently, improved diagnostic techniques have identified various biomarkers that may have an important role in prediction of AF and related outcomes (Tables 1, 2, and 3).

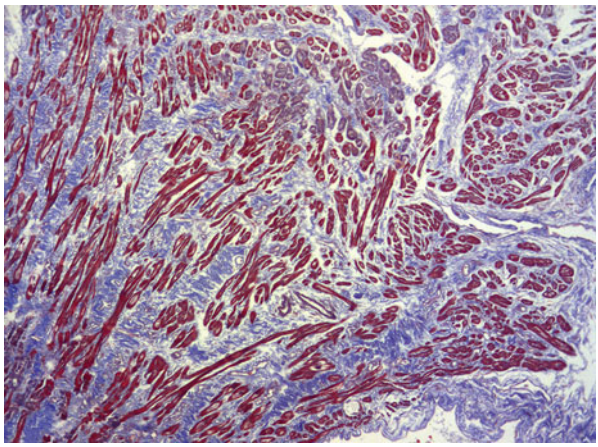

Fig. 1 Connective tissue deposition and myocardial fibrosis in atrial tissue assessed by Masson's Trichrome staining. Magnification ×100

Otherwise, AF confers a prothrombotic or hypercoagulable state (Fig. 2), which participates in the two- to sevenfold increased risk for thromboembolic complications. AF fulfills the Virchow's triad for thrombogenesis, including left atrial blood stasis ("flow abnormalities"), endothelial damage/dysfunction ("vessel wall abnormalities"), and abnormal blood constituents (Watson et al. 2009). Nonetheless, the precise mechanism(s) of how AF results in activation of the coagulation cascade are unclear.

In this setting, biomarkers could take an interesting role. The Food and Drug Administration defines biomarker as any measurable indicator that is potentially useful along the whole spectrum of the disease process; research and development of new therapies; diagnosis, prognosis, and monitoring progression of a disease; or response to treatment (Goodsaid and Frueh 2007). In the last decades, biomarkers have gained huge scientific and clinical value and interest in medical practice. The ideal biomarker should be easily obtained with minimum discomfort or risk to the patient, may also appear or disappear over the course of disease progression, and thus may be useful in determining the prognosis of a disease within an individual. Another biomarker may change as a drug therapy is started, adjusted, or discontinued, ultimately aiding in the monitoring of the patient's response to that particular therapy. In addition, the rapid return of results for early initiation of treatment and monitoring effectiveness is highly desirable. This could be a test performed during a patient's office visit with an immediate result. Finally, a reliable biomarker will have a detection method that could be both sensitive and specific and highly reproducible among clinical laboratories.

Renewed interest has arisen to study the different pathways that underlay this hypercoagulable state in AF. Figure 3 shows different pathways underlying hypercoagulable state in AF and their association with biomarkers (Vilchez et al. 2014).

Table 1 Main studies related with stroke and thromboembolism biomarkers in AF

Study	Year	N	Design	Association	Adjusted score	Main end points
Troponin						
Hijazi et al.	(2012)	6,189	RE-LY trial	HR 1.68 (0.97–2.89); $P = 0.0232$	$CHADS_2$ CHA_2DS_2–VASc	Association with troponin I and CV events
Roldán et al.	(2012)	930	Prospective real-world cohort	HR 2.37 (1.08–5.02); $p = 0.032$	$CHADS_2$	Prognostic value of hsTnT associated with CV events
				HR 2.44 (1.13–5.26); $p = 0.023$	CHA_2DS_2–VASc	
Hijazi et al.	(2014)	14,897	ARISTOTLE trial	HR 1.94 (1.35–2.78); $p = 0.0010$	CHA_2DS_2–VASc	Prognostic value of hsTnT in addition with risk score
NT-proBNP						
Hijazi et al.	(2012)	6,189	RE-LY trial	HR 2.09 (1.22–3.58); $p < 0.0001$	$CHADS_2$ CHA_2DS_2–VASc	Association with NT-proBNP with CV events
Hijazi et al.	(2013)	14,892	ARISTOTLE trial	HR 2.35 (1.62–3.40); $p = <0.0001$	$CHADS_2$ CHA_2DS_2–VASc	Prognostic value of NT-proBNP in AF patients
Roldán et al.	(2014)	1,172	Prospective real-world cohort	HR 2.71 (1.54–4.75); $p = 0.001$	CHA_2DS_2–VASc	Prognostic value of NT-proBNP in clinical practice
Renal function biomarkers						
Glomerular filtration rate (GFR)						
Go et al.	(2009)	10,908	ATRIA substudy GFR <45 ml/min/ 1.73 m^2	HR 1.39 (1.13–1.71); $p = 0.0082$	_____	Additional incremental risk factors for ischemic stroke

(*continued*)

Table 1 (continued)

Study	Year	N	Design	Association	Adjusted score	Main end points
Hohnloser et. al	(2012)	7,518	ARISTOTLE trial GFR <50 ml/min/ 1.72 m^3	HR 0.61 (0.39–0.94); $p = 0.406$	_____	CV outcome relationship with renal function
Roldán et al.	(2013)	978	Prospective real-world cohort	_____	_____	Determine if CKD improves predictive value $CHADS_2$
Roldán et al.	(2013)	978	Prospective cohort GFR <30 ml/min/ 1.73 m^2	HR 1.42 (1.11–1.83); $p = 0.006$	$CHADS_2$	Evaluated renal function on prognosis anticoagulated AF patients
Cystatin C						
Ix et al.	(2007)	990	Prospective study	HR 3.6 (1.8–7); $p < 0.001$	_____	Evaluated association of cystatin C with CV events
Hohnloser et al.	(2012)	7,518	ARISTOTLE trial Cystatin-GFR <50 ml/ min	HR 0.64 (0.39–1.05); $p = 0.098$	_____	Cardiovascular outcomes in renal function
Beta-trace protein (BTP)						
Vílchez et al.	(2013)	1279	Consecutive AF patients	HR 1.31 (1.11–1.56); $p = 0.002$	CHA_2DS_2–VASc	Association of BTP with CV diseases
Thrombogenesis						
Plasma D-dimer						
Roldán et al.	(2011)	829	Prospective real-world cohort	NS	CHA_2DS_2–VASc HAS-BLED	D-dimer levels could refine risk schemes
Eikelboom et al.	(2010)	6,170	RE-LY substudy	HR 2.97 (1.76–5.03); $p = 0.005$	$CHADS_2$	D-dimer could be independent predictor of CV events

Prothrombin fragment 1 +2 (F1+2)						
Feinberg et al.	(1999)	553	SPAF III study	HR 1.9 (0.8–4.9); $p = 0.18$	_____	Relation markers of thrombin with stroke
Tissue plasminogen activator–plasminogen activator inhibitor (tPA–PAI)						
Roldán et al.	(1998)	36	Prospective chronic AF cohort	_____	_____	Relationship of fibrinolytic system with AF
Vene et al.	(2003)	113	Prospective chronic AF cohort	HR 1.09 (1.01–1.179; $p = 0.02$)	_____	Predictive value of hemostatic markers for CV events
Endothelial damage biomarkers						
von Willebrand factor (vWF)						
Roldán et al.	(2011)	829	Prospective real-world cohort	HR 2.71 (1.78–4.13); $p < 0.001$	$CHADS_2$ CHA_2DS_2–VASc	Plasma vWF levels could be used to refine clinical risk scores
Lip et al.	(2006)	994	SPAF III trial	HR 1.61 (0.87–2.96) NS	$CHADS_2$	Additive role of plasma vWF for AF risk stratification
				HR 1.72 (0.94–3.22) NS	BIRMINGHAM	
Roldán et al.	(2005)	200	Prospective cohort of anticoagulation clinic	$r = -0.054$; $p = 0.460$	$CHADS_2$	Risk scores could be related to plasma vWF
				$r = 0.062$; $p = 0.460$	FRAMINGHAM	
E-selectin						
Krishnamoorthy et al.	(2013)	423	Real-world AF community patients	RR 3.7 (2.51–5.31); $p < 0.001$	_____	Relationship between E-selectin and CV events

(*continued*)

Table 1 (continued)

Study	Year	N	Design	Association	Adjusted score	Main end points
Platelets						
CD40 ligand (CD40L)						
Ferro et al.	(2007)	231	Prospective cohort of persistent AF patients	HR 4.63 (1.92–11.20); $p = 0.001$	_____	Assess if CD40L is a predictor of stroke
Lip et al.	(2007)	880	SPAF III trial	$r = -0.096$; $p = 0.02$	$CHADS_2$	Relationship of plasma levels of CD40L and risk of stroke
				pseudo$r^2 = 0.007$; $p = 0.02$	NICE	
P-selectin						
Heeringa et al.	(2006)	162	Rotterdam cohort study	RR 0.90 (0.72–1.13) NS	_____	Association between plasma markers and stroke
Beta-thromboglobulin						
Feinberg et al.	(1999)	1338	SPAF III trial	RR 1 (0.5–2.1) NS	_____	Relationship between BTG and stroke in AF
Inflammation biomarkers						
C-reactive protein (CRP)						
Lip et al.	(2007)	880	SPAF III trial	$r = 0.147$; $p < 0.001$	$CHADS_2$	Relationship of CRP plasma levels with stroke
				pseudo $r^2 = 0.016$; $p < 0.001$	NICE	

Conway et al.	(2004)	77	Prospective chronic AF cohort	HR 2.09 (0.97–4.47); $p = 0.06$	_____	Inflammatory state would be associated with stroke
Interleukin-6 (IL-6)						
Conway et al.	(2004)	77	Prospective chronic AF cohort	HR 2.91 (1.20–6.51); $p = 0.007$	_____	Inflammatory state would be associated with stroke
Roldán et al.	(2012)	930	Prospective real-world cohort	HR 1.97 (1.29–3.02); $p = 0.002$	$CHADS_2$	Assess clinical usefulness of IL-6 for CV events
				HR 1.89 (1.23–2.89); $p = 0.004$	CHA_2DS_2–VASc	
Aulin et al.	(2011)	6,217	RE-LY substudy	HR 2.01 (1.25–3.23); $p = 0.0091$	_____	Relationship between IL-6 and CV events
Adiponectin						
Hernández-Romero et al.	(2013)	918	Prospective stable anticoagulated patients	NS; $p = 0.177$	_____	Plasma adiponectin can be predictive of CV risk
Growth differentiation factor 15 (GDF-15)						
Wallentin et al.	(2014)	14798	ARISTOTLE trial	C index 0.670; $p = 0.0091$	CHA_2DS_2–VASc	Evaluated prognostic value of GDF-15 alone and in addition to other biomarkers

Table 2 Main studies related to mortality with biomarkers in AF

Study	Year	N	Design	Association	Adjusted score	Main end points
Troponin						
Hijazi et al.	(2012)	6,189	RE-LY trial	HR 3.20 (2.20–4.65); $p < 0.0001$	$CHADS_2$	Association with troponin I and CV events
					CHA_2DS_2–VASc	
Roldán et al.	(2012)	930	Prospective real-world cohort	HR 1.79 (1.13–2.83); $p < 0.001$	$CHADS_2$	Prognostic value of hsTnT associated with CV events
				HR 1.99 (1.25–3.20); $p = 0.004$	CHA_2DS_2–VASc	
Van den Bos et al.	(2011)	407	Prospective study	HR 3.77 (1.42–10.02); $p < 0.001$	_____	Troponin I levels could be associated with CV events
NT-proBNP						
Fonorow	(2007)	48,629	ADHERE registry	HR 2.23 (1.91–2.62); $p < 0.0001$	_____	Determine if BNP levels are predictive of in-hospital mortality
Hijazi et al.	(2012)	6,189	RE-LY trial	HR 5.07 (2.95–8.71); $p < 0.0001$	$CHADS_2$	Association of NT-proBNP with CV events
					CHA_2DS_2–VASc	
Hijazi et al.	(2013)	14,897	ARISTOTLE trial	HR 2.25 (1.80–2.81); $p = <0.0001$	CHA_2DS_2–VASc	Prognostic value of NT-proBNP in AF patients in addition to risk score
Roldán et al.	(2014)	1,172	Prospective real-world cohort	HR 1.66 (1.16–2.37); $p = 0.006$	CHA_2DS_2–VASc	Prognostic value of NT-proBNP in clinical practice
Renal function biomarkers						
Glomerular filtration rate (GFR)						
Go et al.	(2004)	1,120,295	Prospective cohort	HR 3.2 (3.1–3.4)	_____	Independent association between GFR and death
			GFR 15–29 ml/min/1.73 m^2	HR 5.9 (5.4–6.5)		
			GFR <15 ml/min/1.73 m^2			

Hohnloser et al.	(2012)	7,518	ARISTOTLE trial GFR <50 ml/min/1.72 m^3	HR 0.78 (0.63–0.96); $p = 0.319$	_____	CV outcome relationship with renal function
Roldán et al.	(2013)	978	Prospective cohort GFR <30 ml/min/1.73 m^2	HR 1.47 (1.13–1.91); $p = 0.004$	_____	Evaluated renal function on prognosis anticoagulated AF patients
Astor et al.	(2012)	15792	ARIC study GFR <60 ml/min/1.73 m^2	HR 1.57 (1.27–1.93), $p < 0.001$	_____	Association of serum creatinine with mortality and CV events
Cystatin C						
Astor et al.	(2012)	15,792	ARIC study	HR 2.89 (2.32–3.61); $p < 0.001$	_____	Association of cystatin C with death
Hohnloser et al.	(2012)	7,518	ARISTOTLE trial Cystatin-GFR <50 ml/min	HR 1 (0.79–1.26); $p = 0.706$	_____	Cystatin C related to CV outcomes
Beta-trace protein (BTP)						
Vílchez et al.	(2013)	1,279	Consecutive AF patients	HR 2.08 (1.49–2.90); $p = 0.001$	CHA_2DS_2–VASc	Association of BTP with mortality
Astor et al.	(2012)	15,792	ARIC study	HR 1.90 (1.54–2.36); $p < 0.001$	_____	Association of BTP with mortality
Inflammation biomarkers						
C-reactive protein (CRP)						
Lip et al.	(2007)	880	SPAF III trial	HR 1.55 (1.03–2.31); $p = 0.035$	_____	Relationship of CRP plasma levels with death

(*continued*)

Table 2 (continued)

Study	Year	N	Design	Association	Adjusted score	Main end points
Hermida et al.	(2012)	293	ARIC study	HR 2.52 (1.49–4.25); $p < 0.001$	$CHADS_2$	Association of CRP with all-cause and CV death
			All-cause mortality	HR 2.23 (1.04–4.57); $p = 0.10$		
			CV mortality			
Interleukin-6 (IL-6)						
Roldán et al.	(2012)	930	Prospective real-world cohort	HR 2.48 (1.60–3.85); $p = 0.001$	$CHADS_2$	Assess clinical usefulness of IL-6 for CV events
				HR 2.19 (1.36–3.52); $p = 0.001$	CHA_2DS_2–VASc	
Growth differentiation factor 15 (GDF-15)						
Wallentin et al.	(2014)	14,798	ARISTOTLE trial	HR 2.10 (1.62–2.73); $P < 0.0001$	CHA_2DS_2–VASc	Evaluated prognostic value of GDF-15 alone and in addition to other biomarkers

Table 3 Main studies related to major bleeding with biomarkers in AF

Study	Year	N	Design	Association	Adjusted score	Main end points
Troponin						
Hijazi et al.	(2012)	6,189	RE-LY trial	HR 1.89 (1.30–2.75); $p < 0.0040$	$CHADS_2$ / CHA_2DS_2–VASc	Association between troponin I levels and major bleeding
Hijazi et al.	(2014)	14,897	ARISTOTLE trial	HR 1.91 (1.43–2.56); $p = 0.0001$	CHA_2DS_2–VASc	Evaluated prognostic hsTnT values in addition to CHA_2DS_2–VASC and clinical risk factors
NT-proBNP						
Hijazi et al.	(2012)	6,189	RE-LY trial	HR 1.28 (0.87–1.88); $p = 0.5272$ NS	$CHADS_2$ / CHA_2DS_2–VASc	Association of NT-proBNP with CV events
Hijazi et al.	(2013)	14,892	ARISTOTLE trial	HR 1.07 (0.82–1.40); $p = 0.0667$ NS	CHA_2DS_2–VASc	Evaluated if NT-proBNP levels could predict bleeding events
Renal function biomarkers						
Glomerular filtration rate (GFR)						
Hohnloser et al.	(2012)	7,518	ARISTOTLE trial / GFR <50 ml/min/1.72 m^3	HR 0.48 (0.37–0.64); $p = 0.004$	_____	CV outcome relationship with renal function
Roldán et al.	(2013)	978	Prospective cohort / GFR <30 ml/min/1.73 m^2	HR 1.44 (1.08–1.94); $p = 0.015$	_____	Evaluated effect of GFR on bleeding in anticoagulated AF patients
Cystatin C						
Hohnloser et al.	(2012)	7,518	ARISTOTLE trial / Cystatin-GFR <50 ml/min	HR 0.65 (0.47–0.91); $p = 0.775$	_____	Association of cystatin C with CV outcomes

(*continued*)

Table 3 (continued)

Study	Year	N	Design	Association	Adjusted score	Main end points
Beta-trace protein (BTP)						
Vílchez et al.	(2013)	1,279	Consecutive AF patients	HR 1.88 (1.18–3.00); $p = 0.008$	HAS-BLED	Association of BTP with bleeding
Thrombogenesis biomarkers						
Plasma D-dimer						
Eikelboom et al.	(2010)	6,170	RE-LY substudy	HR 2.34 (1.63–3.36); $p < 0.0001$	$CHADS_2$	D-dimer could be independent predictor of CV events
Endothelial damage biomarkers						
von Willebrand factor (vWF)						
Roldán et al.	(2011)	829	Prospective real-world cohort	HR 4.47 (1.86–10.75); $p = 0.001$	$CHADS_2$	vWF could be used to refine major bleeding in AF patients
					CHA_2DS_2–VASc	
					HAS-BLED	
Growth differentiation factor 15 (GDF-15)						
Wallentin et al.	(2014)	14,798	ARISTOTLE trial	HR 2.00 (1.48–2.69); $P < 0.0001$	HAS-BLED	Evaluated prognostic value of GDF-15 alone and in addition to other biomarkers

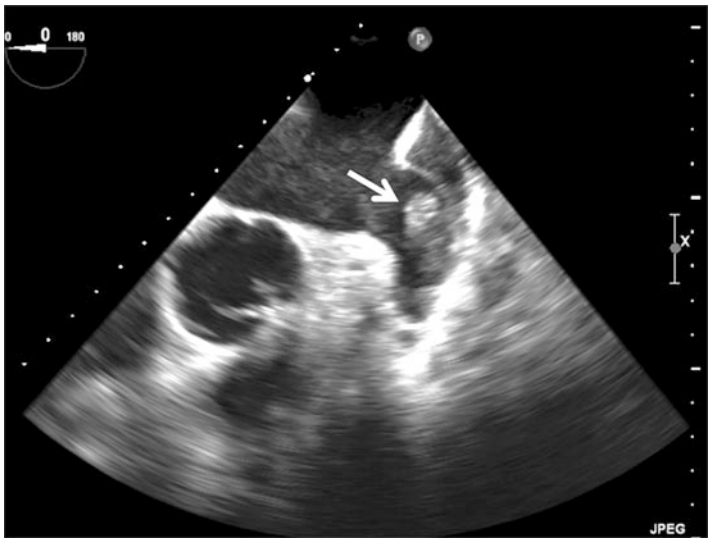

Fig. 2 An image of left atrial appendage thrombus with thansesophageal ecocardiography (Figure obtained from Gonzalo de la Morena for illustrations)

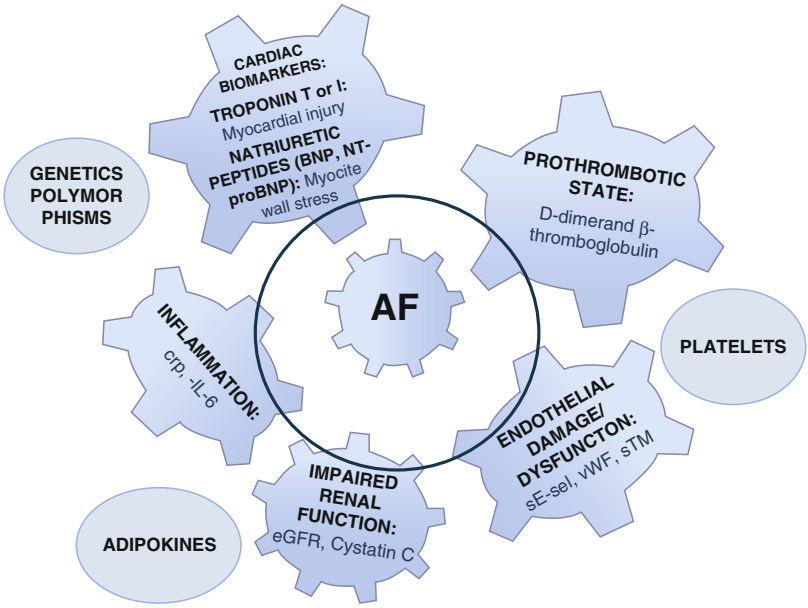

Fig. 3 Different pathways involved in the AF pathophysiology related to various biomarkers (Figure obtained from Vílchez et al. 2014)

Risk Stratification in AF

Oral anticoagulation (OAC) is highly effective in reducing stroke risk and mortality rates in patients with AF, but also increases the risk of bleeding (Singer et al. 2009). Assessment of AF- associated stroke risk is mainly based on clinical risk scores such as $CHADS_2$ and CHA_2DS_2–VASc. The current guidelines on AF recommend the use of CHA_2DS_2–VASc score to assess thromboembolic risk (Craig et al. 2014; Camm et al. 2012). CHA_2DS_2–VASc score assigns 1 point each for presence of congestive heart failure, hypertension, diabetes mellitus, vascular disease, age >65, and sex category (female gender) and 2 points to age >75 years and prior stroke/TIA (Craig et al. 2014). AF patients with CHA_2DS_2–VASc ≥2 should be considered for starting OAC, and patients with CHA_2DS_2–VASc = 1 would have considered indication to initiate OAC for preventing stroke. Subjects categorized to be OAC eligible will be exposed to an increased risk of major bleeding (Providencia et al. 2012). The alternative would be to leave some patients untreated and exposed to the risk of fatal and devastating strokes. Stroke risk is also closely related to bleeding risk, and OAC therapy needs to weigh the benefit from stroke prevention against the bleeding risk. Many thromboembolic risk factors have also been identified as bleeding risk factors (e.g., advanced age or uncontrolled hypertension) (Lip et al. 2011). The HAS-BLED (Hypertension, Abnormal Renal/Liver Function, Stroke, Bleeding History or Predisposition, Labile International Normalized Ratio, Elderly, and Drugs/Alcohol Concomitantly) was proposed as a practical tool to assess the individual bleeding risk of "real-world" AF patients (Pisters et al. 2010). A score of >3 pointes indicates "high risk"; however, this score, as European guidelines state, does not contraindicate OAC therapy (Camm et al. 2012). In these patients with high bleeding risk, close monitoring is required after the initiation of antithrombotic therapy, as well as efforts to correct the potentially reversible risk factors for bleeding.

Although these scores are easy to apply, the clinical risk scores have limited capacity for prediction of thromboembolic events, with low values for the area under the receiver operating characteristic curve, known as the c-statistic. Numerous studies have highlighted the potential utility of biomarkers in enhancing risk stratification and improving the predictive power of clinical risk scores, such CHA_2DS_2–VASc (Hijazi et al. 2012). Advances in genomics, proteomics, and molecular pathology have generated many candidate biomarkers that might play an important role in prediction of related outcomes in AF (López-Cuenca et al. 2010; Hijazi et al. 2013a). There is much interest in blood-based biomarkers that could provide additional refinement to clinical risk stratification.

On the other hand, most of the interest has been focused on embolic risk and the development of major bleeds in patients under OAC. However, an increase on all-cause death in patients with AF has been observed (Camm et al. 2012). It probably merits to assess the risk of death of our patients in clinical practice.

We will review the published data about biomarkers in AF and focus on the predictive ability of the three most important cardiovascular events: thromboembolism, mortality, and major bleeding.

Cardiac Biomarkers and AF Outcomes

Stroke and Systemic Thromboembolism

Cardiac Troponins

Cardiac troponins are intracellular proteins involved in heart muscle contraction, and thus they are known as sensitive and specific biomarkers of myocardial injury (Roldán et al. 2012). Troponins as an indicator of high risk of stroke or systemic embolism were first reported from the Randomized Evaluation of Long-Term Anticoagulant Therapy (RE-LY) biomarker substudy performed in 6,189 patients with AF and treated with either warfarin or dabigatran (Hijazi et al. 2012). The Apixaban for the Prevention of Stroke in Subjects with Atrial Fibrillation (ARISTOTLE) troponin substudy results verified that the troponin levels were related to the risk of stroke, in a continuous fashion, independent of baseline characteristics and other biomarkers (Hijazi et al. 2014a). These two studies, therefore, provide firm evidence that patients classified as having elevated troponin levels based on the 99th percentile upper reference limit for healthy subjects (troponin I >0.04 μg/L or high-sensitivity troponin T >13 ng/L) had significantly increased risk of stroke and systemic embolism independent of clinical characteristics and other powerful biomarkers. Moreover, in comparison with $CHADS_2$ and CHA_2DS_2–VASc, when adding information about troponin measurements to a predictive model for stroke outcomes, the troponin I level provided significant incremental prognostic information.

Interestingly, a recent ARISTOTLE substudy (Hijazi et al. 2014) assessed the distribution and compared and combined the prognostic value of cTnI and cTnT measured with high-sensitivity methods in patients with AF. Their findings show that the correlation between cTnI and cTnT concentrations was moderate and patients with both troponins above the median had significantly higher risk for stroke/systemic embolism than those with both troponins below median over a median 1.9 years of follow-up. However, intermediate risks were observed when only one assay of troponin was above the median; and when considered with information from other clinical risk factors, cTnI and cTnT provide similar prognosis information. Renal impairment was the most important determinant of increased troponin concentrations, with similar influence on both markers.

The underlying cause of the association between high troponin and stroke is not clearly elucidated. Troponin increase could be related to AF per se, or caused by coexistent cardiovascular risk factors, or troponin may simply reflect a sick heart. Even without a complete understanding of the mechanism, the firm evidence and the general availability of cardiac troponin measurements for routine care in most hospitals worldwide make it a very attractive candidate for use to improve prognostication of patients with AF, in addition to the currently recommended clinical stroke risk stratification.

Natriuretic Peptides

B-type natriuretic peptide (BNP) is a neurohormone synthesized by myocytes, predominantly in the left ventricle (LV), in response to increased wall tension such as

volume or pressure overload (Daniels and Maisel 2007). BNP is secreted as an inactive prohormone. It is cleaved in equimolar amounts into the bioactive hormone, BNP, and the inactive N-terminal fragment (NT-proBNP) (Boomsma and van den Meiracker 2001). Although natriuretic peptides are excellent markers of LV function and considered as a simple and effective tool to diagnose heart failure or LV dysfunction, these indices have also been analyzed in different cardiovascular disorders.

Initial studies demonstrated elevated levels of BNP in patients with AF compared with matched controls in sinus rhythm (Ellinor et al. 2005; Shelton et al. 2006). Regarding the influence of these biomarkers, it was not until RE-LY results that the prognostic value of this information was highlighted (Hijazi et al. 2012). In the RE-LY substudy, the levels of NT-proBNP correlated with the risk of thromboembolic events and cardiovascular mortality with higher risk at rising levels. In the larger ARISTOTLE biomarker study was observed an especially strong association between increased risk of ischemic stroke and rising NT-proBNP levels (Hijazi et al. 2013). Recently our group showed that in a real-world cohort of anticoagulated patients with AF, NT-proBNP provided complementary prognostic information to an established clinical risk score (CHA_2DS_2–VASc) for the prediction of stroke/systemic embolism (Roldán et al. 2014).

The use of transesophageal echocardiography provides information of variables associated with thromboembolism, such as dense spontaneous echo contrast, low flow velocities in the left atrial appendage, or even the presence of left atrial thrombus. These parameters have been linked in small studies to elevated levels of natriuretic peptides and may contribute to the prognostic properties of these biomarkers in AF (Igarashi et al. 2001). Okada et al. (2011) showed that BNP levels can serve as a marker for left atrial thrombus in patients with AF who suffered acute ischemic stroke or transient ischemic attack. It has been suggested that the development of new onset AF in patients with acute ischemic stroke was strongly associated with higher BNP levels. This theory proposes that atrial dysfunction is an established risk factor of thrombus formation in AF and thereby represents plausible pathophysiologic mechanism for relation between natriuretic peptides and thromboembolic events in AF. The improved risk prediction by adding natriuretic peptides to clinical risk stratification models is substantial, and the availability of the analysis is widespread and easy accessible. Therefore, the opportunity to use measurements of NT-proBNP to improve risk stratification of AF patients in routine clinical practice is very attractive.

Renal Function Biomarkers in AF

Glomerular filtration rate (GFR) is accepted as useful index of renal function and is usually estimated from serum levels of endogenous filtration markers such as creatinine. The prevalence of AF is higher in end-stage renal disease populations compared with the general population, and the AF prevalence increases when GFR decreases in general chronic kidney disease (CKD) cohorts (Ananthapanyasut et al. 2010; Deo et al. 2010). Concerning renal function and stroke outcomes in AF, Go et al. reported an independent risk increase with reduced GFR or if proteinuria was present (Go et al. 2009). Hohnloser et al. reported similar findings based on

ARISTOTLE trial population, in which increased rates of stroke occurred as renal function was deteriorated (Hohnloser et al. 2012). Remarkably, the recent study published by our group based on c-statistics and the integrated discrimination improvement showed that adding CKD to the stroke risk scores did not independently improve the predictive value of current clinical scores (Roldán et al. 2013a).

Cystatin C is a small protein, synthesized at a constant rate in all nucleated cells (Abrahamson et al. 1990). It is freely filtered by the glomerulus and does not return to the blood flow. Thus, this protein was proposed as a more reliable marker of renal function than serum creatinine, in particular for the detection of small reductions in GFR (Laterza et al. 2002; Newman et al. 1995). Cystatin C is a considered to reflect microvascular renal dysfunction and has been linked to elevated levels of markers of coagulation, raised levels of inflammatory markers, and severity of coronary artery disease (Dubin et al. 2011). Furthermore, cystatin C significantly improves risk stratification compared with creatinine-based estimation of GFR in both elderly and coronary artery disease populations (Ix et al. 2007). The significance of cystatin C in an AF population was recently reported from ARISTOTLE and RE-LY biomarker substudies (Hohnloser et al. 2012). Rising cystatin C levels were independently associated with increased rates of stroke or systemic embolism.

Beta-trace protein (BTP) is a lipocalin glycoprotein identified as lipocalin-type prostaglandin D synthase (Hoffmann et al. 1993). In the human heart, BTP is localized in myocardial cells and atrial and ventricular endocardial cells. BTP levels are elevated in the circulation of patients with severe coronary heart disease. In addition, BTP has been considered as an accurate biomarker of glomerular filtration, perhaps even more accurate than serum creatinine or eGFR in detecting impaired renal function, given that BTP has less dependence on extrarenal factors such as age, body dual-mass index, nutritional status, and sex. Our group demonstrated that the addition of BTP improves the predictive value of a clinical risk score (i.e., CHA_2DS_2–VASc) for the detection of thrombotic events (Vílchez et al. 2013). Thus, BTP may be another novel predictor of thromboembolism.

Thrombogenesis Biomarkers in AF

Plasma D-dimer is a fibrin degradation product and is a marker of intravascular thrombogenesis and fibrin turnover. Levels of D-dimer are elevated compared with matched controls in sinus rhythm and even seem to remain increased despite successful cardioversion (Asakura et al. 1992). Levels of D-dimer further seem to rise along with the accumulation of clinical risk factors for thromboembolism or by the presence of atrial appendage thrombi. The recent trials (RE-LY, ARISTOTLE) describe a significant association between baseline D-dimer levels and the risk of stroke independent of established risk factors including CHA_2DS_2–VASc variables (Eikelboom et al. 2010).

The risk increases with higher D-dimer levels as evidenced by a threefold increase of stroke or systemic embolism when the top vsersus bottom quartiles were compared. These results suggest that D-dimer may also be a clinically useful risk marker in AF. However, a study realized for our group did not find that D-dimer levels in an anticoagulated AF cohort were related to the prognosis (Roldán et al. 2011).

Prothrombin fragment 1 +2 (F1+2) reflects in vivo thrombin generation, is reported to be elevated in AF, and is suppressed by anticoagulation in a dose-dependent manner. F1+2 levels were independently associated with advanced age, female sex, systolic blood pressure, and heart failure and were not influenced by aspirin use. In the third Stroke Prevention in Atrial Fibrillation (SPAF III) study, elevated F1+2 levels, as index of thrombogenesis, were associated with a clinical risk factor for stroke in AF (Feinberg et al. 1999). Moreover, F1+2 levels measured were higher in participants who subsequently suffered thromboembolic events, but differences were only marginally statistically significant.

Fibrinolytic system dysfunction may contribute to increase risk of thrombosis. Plasma levels of modified antithrombin III (ATM), tissue plasminogen activator (tPA), its inhibitor (PAI-1), tPA–PAI-1 complexes, and plasmin–antiplasmin (PAP) complexes have been measured in plasma from patients with chronic atrial fibrillation compared with healthy subjects. The results showed a hypofibrinolytic state caused by elevated PAI-1 levels with no increase in PAP complex concentration (Roldán et al. 1998). Vene et al. observed that high levels of tPA antigen levels were significantly associated with combined cardiovascular events in AF patients (Vene et al. 2003). In conclusion, high levels of D-dimer and tPA antigen during oral anticoagulant therapy may be associated to combined cardiovascular events in AF patients and, on this basis, could be useful additional markers of cardiovascular risk in such patients.

Endothelial Damage Biomarkers

Plasma levels of von Willebrand factor (vWF), soluble thrombomodulin (sTM), and soluble E- selectin (sE-sel) are used as indexes of damage/dysfunction, endothelial damage, and endothelial activation. Indeed, vWF is an established biomarker of endothelial damage/dysfunction, as it is synthesized by vascular endothelial cells and promotes platelet adhesion and aggregation, leading to thrombus formation (Roldán et al. 2011). Plasma vWF levels have been associated with independent risk factors for stroke (heart failure, previous stroke, age, and diabetes) and stroke risk stratification schemes (Conway et al. 2004). Plasma vWF levels correlated with two risk stratification scores for stroke ($CHADS_2$ and Framingham) in AF patients (Lip et al. 2006). Recent studies showing an association between AF and endothelial damage/dysfunction found that it is reversed after restoration of sinus rhythm by catheter ablation or electrical cardioversion. Despite the immediate improvement of endothelial function after sinus rhythm restoration, more sustained postcardioversion (delayed) injury and shedding of endothelial cells could be contributors to longer-term thromboembolic complications (Freestone et al. 2006). These changes in the hypercoagulable state and endothelial (dys)function occur within minutes of acute AF onset and appear to be persistent after sinus rhythm restoration. Roldán et al. confirmed that an increased plasma vWF levels were associated with adverse prognosis on "real-life" AF patients, mainly thrombotic events (Roldán et al. 2005). Therefore, the addition of vWF as a biomarker risk factor may help to refine these clinical risk stratification schemes for stroke.

A soluble form of thrombomodulin is a recognized marker of endothelial dysfunction and may contribute to the hypercoagulable state in AF. Plasma sTM levels are lower in patients with persistent AF (Freestone et al. 2007).

Freestone et al. hypothesized that endothelial dysfunction exists in AF and that this could be demonstrated by impaired flow-mediated dilatation (FMD) and related to plasma indices of endothelial damage/dysfunction plasma biomarkers, as well as total body nitrate/nitrite product (NOx, a measure of endothelial nitric oxide production) (Freestone et al. 2008). This study demonstrated that endothelial dysfunction, as demonstrated by impairment of FMD and raised vWF and E-selectin, is present in AF. Other studies reported that high plasma vWf and sE-sel levels are associated with an increased risk of ischemic stroke in "real-world" patients with AF with a median follow-up of 19 (9–31) months (Krishnamoorthy et al. 2013). These soluble biomarkers may potentially aid clinical risk stratification in this common arrhythmia.

Platelets

Different platelet activation markers have been described in AF patients. However, many abnormal changes in platelets seen in AF could simply indicate underlying vascular comorbidities (Watson et al. 2009). It is uncertain if platelet activation might simply reflect the associated comorbidities with AF (e.g., hypertension and vascular disease) rather than be related to the prothrombotic state in AF per se. For example, Ferro et al. suggested that enhanced platelet activation might even play a role in clinical progression of AF because high soluble CD40L level was predictive for vascular events (stroke and myocardial infarction) in patients with AF (Ferro et al. 2007; Lip et al. 2007). In the Rotterdam Study, plasma-soluble P-selectin levels did predict clinical adverse outcomes in AF, suggesting a role of platelets in the prothrombotic state associated with this disorder (Heeringa et al. 2006). Also, platelet size, measured as mean platelet volume, has been associated with platelet reactivity and as an independent risk factor for future stroke and myocardial infarction.

Beta-thromboglobulin is a platelet-specific protein that indicates platelet activation and is released from alpha-granules during platelet aggregation and subsequent thrombus formation. In the SPAF III study have been shown that BTG levels were not predictive of thromboembolic events either when analyzed as a continuous variable or when those with BTG levels <42 ng/mL were compared with others (Feinberg et al. 1999).

Inflammation Biomarkers

C-reactive protein (CRP) is an established biomarker linked to inflammation and is predominantly synthesized in hepatocytes as an acute-phase reactant. CRP has been frequently studied in cardiovascular diseases and AF. In a small study, Conway et al. reported the association between CRP and a composite outcome of stroke and death in AF (Conway et al. 2004).

Interleukin-6 (IL-6) is circulating cytokine produced by monocytes, macrophages, T lymphocytes, and endothelial cells. IL-6 is the inflammation marker best

related to AF that can induce a prothrombotic state (Kerr et al. 2001). Conway et al., based on a small study, reported the association between IL-6 and a composite outcome of stroke and death. Recently, our group showed that high IL6 and high TnT remained significantly associated with stroke/TIA or systemic embolic even after adjusting for $CHADS_2$ score (17). Preliminary results from RE-LY biomarker substudy (Aulin et al. 2011) showed that in patients with top quartile levels compared with the bottom quartile, there was a doubling of stroke risk in adjusted analysis. These findings suggest that IL-6 may potentially be used to refine clinical risk stratification in AF.

Other Biomarkers

Adiponectin presents anti-inflammatory, atherogenic, and antihypertrophic functions, and both of them have been associated with multiple known risk factors for AF, including inflammation, diabetes, obesity, myocardial infarction, and incident heart failure (Rienstra et al. 2012). Hernández-Romero et al. found how low levels of adiponectin were independently associated with adverse cardiovascular events but only in female AF patients, and the lack of association in men could be because of testosterone decreasing adiponectin production (Hernández-Romero et al. 2013). These dates confirmed the importance of AF as a risk marker of atherosclerotic vascular damage, and adiponectin could exert a protective role against cardiovascular diseases.

Growth differentiation factor 15 (GDF-15) is a divergent member of the transforming growth factor-β family that can be secreted from a broad range of cells, for example, cytokine secreted from adipocytes and myocytes in response to, and may be protecting against, stress such as cellular ischemia and mechanical and oxidative stress. In the last months it has been described that the prognostic information provided by GDF-15 was independent of clinical characteristics and clinical risk scores (Wallentin et al. 2014). However, after adjustment for the other cardiac biomarkers, the prognostic value for stroke attenuated.

Mortality

Cardiac Troponins

These markers of myocardial injury and stress also play a relevant role in prediction of mortality in patients with AF. In routine daily practice, minor troponin elevation below the 99th percentile in patients with AF is attributed to the rapid or irregular ventricular response and does often not undergo stress testing and coronary angiography. Van der Bos demonstrated that troponin I release might detect additional or ongoing myocardial damage, ultimately leading to deterioration in cardiac function in patients hospitalized for atrial fibrillation, which constituted the first study that reported circulation troponin I levels were associated with mortality and major adverse cardiac events in AF (Van den Bos et al. 2011).

Later, in a substudy from the RE-LY trial, risk assessment for cardiovascular death was independently improved when troponin I was added to thromboembolic risk scores (Hijazi et al. 2012). These results were confirmed in the study by Roldán et al., in a stable and chronic anticoagulated AF cohort, whereby increased plasma troponin T levels were associated with an adverse prognosis in AF patients, with regard to cardiovascular events and mortality (Roldán et al. 2012). Recently, an ARISTOTLE substudy observed that the risk of cardiac death and myocardial infarction is highest in patients with increased concentrations of both troponins (Hijazi et al. 2014). Isolated increases of troponin I concentrations generally seemed to be associated with higher risk of cardiac death and myocardial infarction compared with isolated increases in troponin T (Hijazi et al. 2014). Despite the unknown exact underlying pathophysiological mechanism, as discussed above, therapies aimed at reducing ventricular rate, wall stress, or microperfusion might be useful to improve prognosis in the future.

Natriuretic Peptides

The role of natriuretic peptides as powerful prognostic markers for mortality was initially established in heart failure, thereafter in patients with acute coronary syndromes and later in stable coronary artery populations and in asymptomatic community-based elderly subjects (Frustaci et al. 1997).

In the RE-LY substudy (Hijazi et al. 2012), despite adjustment for known risk factors, the risk for cardiovascular mortality was fivefold higher in patients with the highest quartile levels of NT-proBNP in comparison with patients with normal NT-proBNP levels. Similar results have been shown in ARISTOTLE trial (Hijazi et al. 2014), which found improved risk stratification with NT-proBNP, doubling the risk of death. Thus, the addition of NT-proBNP to the risk stratification models resulted in significant improvements in the discrimination performance for mortality and cardiovascular events. Recently, our group showed NT-proBNP was also predictive of all-cause mortality, suggesting that this biomarker may potentially be used to refine clinical risk stratification in anticoagulated patients with AF (Roldán et al. 2014). So, NT-proBNP could give us valuable information about risk of death in patients with AF.

Renal Function Biomarkers

Renal impairment has been associated with an increased risk of death and adverse cardiovascular events in patients with coronary artery disease as well as in the general population (Go et al. 2004). Importantly, renal function may impair in AF patients; so, Roldán et al. showed a decreased eGFR >10 ml/min/1.73 m^2 in 21 % of patients, with one fifth of followed-up patients developing severe CKD (<30 ml/min/1.73 m^2) (Roldán et al. 2013). This study also demonstrated that the presence of impaired renal function was also associated consistently with the development of adverse cardiovascular events and mortality, even after adjusting for the $CHADS_2$ score.

Cystatin C levels were independently associated with increased rates of major bleedings. The significance of cystatin C in AF population was recently reported on ARISTOTLE (Hohnloser et al. 2012) and RE-LY substudies.

On the other hand, BTP, a previously proposed as renal damage biomarker, could also be a predictor of mortality in patients with AF. Astor et al. compared the associations of BTP and other biomarkers with risks of mortality, coronary heart disease, heart failure, and chronic kidney disease and found higher BTP levels with decreasing eGFR and a significant P hazard ratio associated with the studied risk factors (Astor et al. 2012). In contrast, our results show how BTP levels are only slightly dependent upon renal function and, thus, justify the independent prognostic value of plasma BTP levels in patients with AF (Vílchez et al. 2013).

Inflammation Biomarkers

The prognostic value of CRP to all-cause mortality and a composite of ischemic stroke, myocardial infarction, or vascular death was displayed in a larger cohort based on the Stroke Prevention in Atrial Fibrillation III (trial) (Lip et al. 2007).

In a substudy of ARIC cohort, Hermida et al. (69) confirmed the results on hsCRP as a predictor of mortality with significant improvement on the CHA_2DS_2–VASc score by addition of this biomarker. Preliminary results from RE-LY biomarker substudy (Hijazi et al. 2012) showed that C-reactive protein quartile levels remained independently associated with cardiovascular mortality after multivariable adjustments.

Our group has demonstrated raised IL-6 levels in AF, which suggest the presence of an inflammatory state, although this fact appears to be related to clinical variables of the patients, rather than to the presence of AF per se. IL-6 provided prognostic information that was complementary to clinical risk scores for prediction of long-term cardiovascular events and death (Roldán et al. 2012). Thus, IL-6 was also independently and incrementally associated with cardiovascular mortality.

So, both inflammatory biomarkers, as CRP and IL-6, seem to be independent markers for mortality in patients with AF.

Growth Differentiation Factor 15

Plasma levels of GDF-15 are increased in response to inflammation and may be involved in maintaining the inflammatory activity. These experimental data and the results from this and other clinical studies suggest a link – protective or harmful – between GDF-15 and cellular stress as supported by the associations with age, diabetes mellitus, renal disease, smoking, congestive heart failure, and biomarkers of cardiac and renal dysfunction and inflammation. The understanding of the GDF-15 is limited because its receptor and the involved signaling pathways are unknown. Currently, the level of GDF-15 may be interpreted mainly as an integrative signal of severity of disease in several different pathological conditions.

The prognostic role of GDF-15 was presented as a substudy of the ARISTOTLE trial (64); GDF-15 was shown to be an additive prognostic marker for death, even after adjusting for clinical variables, risk factors, and CHA_2DS_2–VASc score and biomarkers (including troponin I, proBNP, cystatin C).

Major Bleeding

Cardiac Troponin

Various studies in acute coronary syndrome populations linking peak troponin I levels to subsequent increase bleeding rate. The Randomized Evaluation of Long-Term Anticoagulation Therapy (RE-LY) substudy also documents an association between elevated troponin I levels and risk of major bleeding (Hijazi et al. 2012). The causality is unknown, but elevated troponin I levels might contribute to the identification of a more fragile AF subpopulation more likely to bleed during anticoagulation.

On the other hand, isolated increases of troponin T displayed a stronger association with major bleeding events according to International Society on Thrombosis and Hemostasis criteria (Hijazi et al. 2014).

Natriuretic Peptides

Several studies (Hijazi et al. 2012, 2013) showed that there was no significant association between NT-proBNP levels and major bleeding. Higher baseline NT-proBNP concentration was strongly associated with each of the major clinical outcomes explored (like stroke or mortality), except major bleeding, even after adjustment for multivariable model.

Renal Function Biomarkers in AF

Major bleeding constitutes a major problem as patients with renal dysfunction currently tend to be undertreated with oral anticoagulation therapy due to the associated higher bleeding risk especially when treaded with vitamin K antagonist therapy (Piccini et al. 2009).

Renal dysfunction has been associated with an increased risk of bleeding (Hijazi et al. 2014; Santopinto et al. 2003); the incidence of major bleeding increased significantly with the impair of renal function. The association of a low eGFR and bleeding events was not surprising, and several studies have reported an association between a low GFR and a significantly increased risk of bleeding in patients with AF taking oral anticoagulation. Thus, renal impairment is an established risk factor for bleeding and has been included in the HAS-BLED bleeding score. Renal dysfunction may be associated with labile international normalized ratio (INR) and worse the average percentage of the time in the optimal therapeutic INR (TTR).

Cystatin C has also been studied related to risk of bleeding in the study by Hohnloser et al. (2012). They showed that high levels of cystatin C, when added to the eGFR equation, were associated with increased HR rates of major bleeding in patients taking warfarin. Although cystatin C achieved improved stroke risk stratification, creatinine-based estimates of renal function were better indicators of the risk of bleeding during OAC treatment in the ARISTOTLE study (Hijazi et al. 2014).

BTP also improved the predictive value of the HAS-BLED score for major bleeding. Of note, a recent analysis showed that the HAS-BLED score has already been shown to perform as well as a multivariate model for predicting major bleeding in patients with AF receiving anticoagulation treatment (Vílchez et al. 2013).

Endothelial Damage Biomarkers

High plasma vWF levels were also an independent predictor of major bleeding in anticoagulated permanent AF patients. These data were confirmed by Roldan et al. whereby an increased plasma vWF levels were associated major bleeding (Roldán et al. 2011). A high plasma vWF level showed an additive effect on the HAS-BLED score, for an intermediate-risk category for bleeding (HAS-BLED score 1–2 points), so that high plasma vWF levels changed the annual risk of a hemorrhagic event from 1.2 % (base on clinical criteria) to 4.7 %.

Thrombogenesis Biomarkers in AF

DD levels at baseline, regardless OAC, were related to major bleeding. These results were confirmed in the RE-LY or ARISTOTLE biomarker substudies, which described an association between DD levels and the risk of major bleeding outcome independent of established risk factors including the $CHADS_2$ variables.

Growth Differentiation Factor 15

In a new study published in *Circulation*, GDF-15 was shown to be an additive prognostic marker for major bleeding in patients with AF receiving oral anticoagulation (Wallentin et al. 2014). The prognostic value for major bleeding remained even in the presence of NT-proBNP and high-sensitivity troponin I.

In this moment, there are no studies that show significant association between circulating adiponectin levels and major bleeding.

Potential Applications to Prognosis, Other Diseases, or Conditions

Several risk stratification scores have been developed to aid decision-making for thromboprophylaxis, which are currently in use and have limited capacity for prediction of thromboembolic events with low values for area under the receiver operating characteristic curve, known as c-statistic. The identification of new biomarkers could, therefore, provide an established clinical risk score like the CHA_2DS_2–VASc score with complementary prognostic information. However, the biomarker should not be expected to improve the identification of those patients with AF who will benefit from OAC. Biomarkers might be helpful in calculating the risk of major bleeding, by providing information on how to select patients who will derive the most benefit from reducing the composite end point, which includes both embolic and bleeding episodes. Tailoring different antithrombotic options to individuals based on biomarker expression has not been explored in patients with AF, but could be an interesting hypothesis for future trials (Marín et al. 2015). In Fig. 2 we may observe the past, present, and future role of biomarkers in AF.

Although several studies have demonstrated the usefulness of adding blood biomarkers to established clinical risk scores, the application of this approach to the daily clinical practice still remains uncertain. Indeed, some evidence for these

biomarkers has been obtained from recent anticoagulation trials (which often have specific inclusion/exclusion criteria, leading to a selected trial cohort being studied), though the evidence for additive value of biomarkers from large non-anticoagulated "real-world" cohorts is more limited.

Summary Points

- This chapter focuses on the biomarkers in relation to atrial fibrillation.
- Atrial fibrillation is the most common sustained cardiac arrhythmia which is associated with high risk of stroke, thromboembolism, and mortality.
- The prevalence AF increases with age. It is projected to increase in the coming decades.
- The underlying mechanisms behind AF describe multiple pathological states leading to various remodeling processes in atrial myocardium.
- The use of OAC significantly reduces the risk of stroke, thromboembolism, and all-cause mortality.
- Improved diagnostic techniques have identified various biomarkers that might play an important role in prediction of AF and related outcomes (stroke, thromboembolism, and mortality).
- The ideal biomarker should be easily obtained with minimum discomfort or risk to the patient, may also appear or disappear over the course of disease progression, and may thus be useful in determining the prognosis of a disease within an individual.
- This would offer opportunities for personalized medicine and focused therapeutic approaches.

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Biomarkers for Abdominal Aortic Aneurysm 24

Demetrios Moris, Antonios Athanasiou, Spiridon Vernadakis, and Sotirios Georgopoulos

Contents

Abstract

Abdominal aorta aneurysm (AAA) is a serious threat for human life, especially in such cases when it is asymptomatic until aneurysm rupture, which is a general cause of death in AAA subjects. The aim of the present chapter, firstly, is to give a conceptual description of the potential biomarkers that can correlate and predict the natural history of an AAA. Secondly, the aim of this chapter is to summarize

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_4

the developments in the literature concerning the novel biomarkers and their potential screening and therapeutic values. In conclusion, currently no specific laboratory markers allow to screen for the disease and to monitor its progression or the results of treatment. Further studies and studies in larger patient groups are required in order to validate biomarkers as cost-effective tools in the AAA disease.

Keywords
Abdominal aortic aneurysms (AAA) • Biomarkers • Rupture • Growth • Circulation • Progression • Screening

Key Facts of Abdominal Aortic Aneurysm Rupture (AAA Rupture)

- An AAA is a bulging, weakened area in the wall of the aorta resulting in an abnormal widening greater than 50 % of the vessel's normal diameter.
- Rupture is the most common complication of AAA.
- The majority of AAAs rupture into the retroperitoneal cavity, resulting in the classical triad of pulsatile mass, pain, and hypotension.
- The only curative treatment for abdominal aneurysm rupture is the emergency operation.
- The operative repair of abdominal aneurysm rupture is associated with high mortality rate.

Definitions

Abdominal aortic aneurysm is a localized dilatation of the abdominal aorta exceeding the normal diameter by more than 50 %.

Rupture is the most common complication of AAA and one of the most fatal surgical emergencies; it has an overall mortality rate of approximately 90 %. Existing evidence indicates better overall outcome when repair of AAA is performed on elective basis compared to the emergency repair.

Endovascular aneurysm repair (or endovascular aortic repair) (EVAR) is a type of endovascular surgery used to treat an abdominal aortic aneurysm The procedure involves the placement of an expandable stent graft within the aorta to treat aortic disease without operating directly on the aorta.

Endoleak is a leak into the aneurysm sac after endovascular repair. There are five types of endoleaks.

Screening for AAA would most benefit those who have a reasonably high probability of having an AAA that is large enough or will become large enough to benefit from surgery. Ultrasonography has a sensitivity of 95 % and specificity of nearly 100 % when performed in a setting of screening for AAA.

Molecular biomarker can be defined as a detectable cell, protein, peptide, gene, or metabolic product that represents biologic processes that take place in an organism at a given time.

Elastase is a protease associated with the breakdown of aortic elastin.

Cathepsins are elastolytic enzymes that expressed strongly in the AAA wall and favor inflammation in AAA lesions by promoting microvascularization and smooth muscle cell apoptosis as well as leukocytes adhesion and proliferation.

Cystatin C is an endogenous inhibitor of cysteine protease activity.

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that are capable of degrading all kinds of extracellular matrix proteins, but also can process a number of bioactive molecules.

Introduction

Abdominal aortic aneurysm (AAA) is a relatively common and potentially life-threatening disease, with a prevalence ranging from 4.1 % to 11.5 % in European populations (Cornuz et al. 2004). Abdominal aortic aneurysm (AAA) has a complex pathophysiology, in which both environmental and genetic factors play important roles (Table 1). The most feared complication of AAA disease is rupture, which often leads to the patient's death (Table 2). While small aneurysms may rupture too, the risk is greater for bigger aneurysms, so early diagnosis through screening and prompt evaluation of which aneurysms are likely to rupture are key points in the management and research around AAA (Table 3). No effective conservative treatment exists, and when decided to intervene, surgical correction remains the only effective and "finite" treatment of AAA, with the optimal timing for surgery being the main debatable point. Existing evidence indicates better overall outcome when repair of AAA is performed on elective basis compared to the emergency repair (Harris et al. 2006; Dueck et al. 2004). Thus screening programs of AAA have been initiated in developed countries such as the UK. Current guidelines recommend elective repair for aneurysms of more than 5.5 cm in diameter, while increased risk groups (women, smokers, vascular hypertension, and chronic airway patients) may

Table 1 AAA pathophysiology

Stages of development	Histological feature	Pathological mechanisms
Initiation	Dilation	Elastin and collagen degradation, inflammation
Progression	Continued extracellular matrix destruction	Angiogenesis and factors important initiation
Rupture	Aortic medial and adventitia tear	Angiogenesis

Table 2 Abdominal aortic aneurysm size and estimated annual risk of rupture

AAA diameter (cm)	Rupture risk (%/year)
<4	0
4–5	0.5–5
5–6	3–15
6–7	10–20
7–8	20–40
>8	30–50

AAA abdominal aortic aneurysm

Table 3 Factors affecting risk of abdominal aortic aneurysm rupture

	Low risk	Average risk	High risk
Diameter	<5 cm	5–6 cm	>6 cm
Expansion	<0.3 cm/year	0.3–0.6 cm/year	>0.6 cm/year
Smoking/COPD	None, mild	Moderate	Severe/steroids
Family history	No relatives	One relative	Numerous relatives
Hypertension	Normal blood pressure	Controlled	Poorly controlled
Shape	Fusiform	Saccular	Very eccentric
Wall stress	Low (35 N/cm^2)	Medium (40 N/cm^2)	High (45 N/cm^2)
Sex	. . .	Male	Female

COPD chronic obstructive pulmonary disease

benefit by a lower threshold of 5 cm. The Operative Mortality Risk of Open Repair of Abdominal Aortic Aneurysm is presented in Table 4.

Traditional AAA screening, evaluation, and surveillance programs employ the use of imaging techniques such as CT angiogram (CTA), ultrasound (sonography) (US), or magnetic resonance imaging (MRI). Despite the proven efficacy of these imaging techniques, the cost associated with such programs can incur significant financial burdens to the health care systems (Lederle et al. 2000; Brady et al. 2004), so alternative methods are continuously being researched.

Biomarkers have attracted much attention in the field of aneurysm research and are defined as measurable molecules (peptides, proteins, metabolic products) that express a specific biological process in the organism at a given time (Becker 2007). Historically, a number of precise definitions of biomarkers can be found in the literature and they fortunately overlap considerably. In 1993, the World Health Organization (WHO), in a report on the validity of biomarkers in environment risk assessment, stated that a definition of biomarkers includes "almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction." In 1998, the National Institutes of Health Biomarkers Definitions Working Group defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Gj 2001). In 2001, the International

Table 4 Operative mortality risk of open repair of abdominal aortic aneurysm

Lowest risk	Moderate risk	High risk
Age <70 years	Age 70–80 years	Age 80 years
Physically active	Active	Inactive, poor stamina
No clinically overt cardiac disease	Stable coronary disease; remote MI; LVEF >35 %	Significant coronary disease; recent MI; frequent angina; CHF; LVEF <25 %
No significant comorbidities	Mild COPD	Limiting COPD; dyspnea at rest; O_2 dependency; FEV_1 < 1 L/s
. . .	Creatinine 2.0–3.0 mg/dL	. . .
Normal anatomy	Adverse anatomy or AAA characteristics	Creatinine >3 mg/dL
No adverse AAA characteristics	. . .	Liver disease (↑ PT; albumin <2 g/dL)
Anticipated operative mortality, 1–3 %	Anticipated operative mortality, 3–7 %	Anticipated operative mortality, at least 5–10 %; each comorbid condition adds ∼3–5 % mortality risk

AAA abdominal aortic aneurysm, *CHF* chronic heart failure, *COPD* chronic obstructive pulmonary disease, *FEV_1* forced expiratory volume in 1 s, *LVEF* left ventricular ejection fraction, *MI* myocardial infarction, *PT* prothrombin time

Programme on Chemical Safety, in coordination with the WHO, the United Nations, and the International Labor Organization, defined a biomarker as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" (Gj 2001).

In order for a biomarker to be useful, it should be able either to detect the disease itself or express its progression. So a useful biomarker should detect the presence of a subclinical aneurysm or be a measure of its size and expansion rate, thus predicting the risk of rupture. It is known that this risk increases when the observed expansion rate is greater than the expected (Limet et al. 1991). Furthermore a biomarker could define the optimal surveillance intervals and possibly identify pathogenic pathways which could guide monitoring and treatment (Urbonavicius et al. 2008). We should not fail to mention that in order for a biomarker to be employed in modern healthcare system, its use should be cost-effective. Some of the patients selected through this process should be pointed toward more focused screening by specialized imaging techniques. Circulating biomarkers thus present as attractive alternatives for screening and monitoring purposes particularly in healthcare systems which lack the infrastructure to support other primary or secondary screening programs.

In this chapter, firstly, the aim is to give a conceptual description of the potential biomarkers that can correlate and predict the natural history of an AAA. Secondly, the aim is to summarize the developments in the literature concerning the novel biomarkers and their potential screening and therapeutic values.

Circulating Biomarkers for Abdominal Aortic Aneurysm

Plasmin, Plasmin Activators, and the Fibrinolytic System

Cysteine, serine, and metalloproteinase systems all have been reported to be involved in the matrix degradation of the aortic wall, causing AAA. Plasmin is a common activator and could be involved in the pathogenesis of AAA by activating all three systems. Plasmin is formed from plasminogen and this process is regulated by the balance between plasminogen activators (tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA), and plasminogen activator inhibitor type 1 (PAI-1). However, when it reaches the circulation, plasmin is immediately inactivated by antiplasmin, forming plasmin–antiplasmin (PAP) complexes. Lindholt et al. (2001a) measured PAP and found a significant positive modest correlation with growth rate and a potential of predicting cases requiring surgery within the first 5 years, especially in combination with the initial AAA size, sensitivity, and specificity both at 83 %. They also studied the activators of plasmin, and surprisingly, it was tPA that trigger this association with a significant and positive correlation between tPA and expansion rate (Lindholt et al. 2001a). A study demonstrated a significant elevation of tPA concentration in plasma in AAA patients compared with controls, which is consistent with previous histologic studies. Reilly et al. (1994) found high levels of tPA in aneurysmal aortas compared with normal aortas and occlusive aortas (Reilly et al. 1994), whereas Shireman et al. (1997) found a similar threefold elevation of tPA in aneurysmal and occlusive aortas compared with normal aorta (Shireman et al. 1997). Lindholt et al. (2003b) found a positive correlation between plasma levels of tPA and the annual AAA expansion rate as well as with serum cotinine, suggesting that smoking may interact with this pathway (Lindholt et al. 2003b). The finding of elevated tPA, in contrast to tPA/PAI-1 complex (Wilson et al. 2008a) in plasma among patients with screening-detected AAA, supports the hypothesis that the fibrinolytic system may be important in the early pathogenesis of AAA (Wanhainen et al. 2007).

Relationships between blood coagulation, fibrinolysis, and morphology of aneurysms were investigated by Yamazumi et al. (1998) who reported that the size of AAA was associated with blood levels of fibrinolytic factors such as D-dimer, fibrinogen/fibrin degradation products, and plasmin inhibitor–plasmin complexes. In a meta-analysis, it is also suggested that plasma fibrinogen and D-dimer concentrations may be higher in patients with AAA than those in subjects without AAA (Takagi et al. 2009a). D-dimer levels are most tightly associated with AAA status. However, D-dimer levels may mediate the observed elevation in acute-phase reactants (Parry et al.). As far as tissue inhibitor of the metalloproteinases-1 (TIMP-1) is concerned, it controls, among others, the activity of MMPs. Although plasma concentrations of TIMP-1 were found to be significantly higher in AAAs than in healthy controls (Nakamura et al. 2000), TIMP-1 levels were found to be lower in AAA wall tissue compared to healthy aortic tissue (Wilson et al. 2005, 2006). Furthermore, TIMP-1 was negatively correlated with MMP-9 (Speelman et al.). Normally, TIMP-1 regulates the activity of MMP-9. This regulation may be

disturbed in patients with AAA, resulting in a lower TIMP-1 concentration for higher MMP-9 levels (Speelman et al.). TIMP-1 and a1-AT were also negatively correlated (Speelman et al.). Recently, alpha 1-antitrypsin (a1-AT), an inhibitor of serine proteases such as trypsin and leukocyte elastase, was correlated with recent AAA growth (Vega de Ceniga et al. 2009). Increased serum a1-AT has been associated with the future development of AAA and has been correlated with AAA expansion but not size (Lindholt et al. 2000; Vega de Ceniga et al. 2009). The pathophysiological meaning is not well established, but it is shown that both MMP-9 and a1-AT are positively correlated with AAA growth (Vega de Ceniga et al. 2009; Lindholt et al. 2000; Takagi et al. 2009c). Hence, serum a1-AT concentration remains a possible biomarker of AAA growth.

Inflammation Interactions with AAA Pathogenesis

Inflammation has emerged as a key process in the pathogenesis of AAA. This has encouraged elucidation of the role of inflammatory cytokines in aneurysm disease. The current knowledge of aneurysm pathophysiology suggests that inflammatory cells within aneurysm wall produce cytokines that stimulate proteolytic enzymes such MMPs (Kishimoto et al. 1995). These enzymes promote the activation and release of cytokines, generating chronic inflammation and extracellular matrix degradation that is the pathologic hallmark of aneurysms. These interactions are dominated by profound activation of the NF-kappaB and AP-1 pathways, hyperexpression of interleukin-6 (IL-6) and IL-8, and neutrophil involvement. Discordant findings for interferon gamma, cytotoxic T cells, B cells, and plasma cells challenge a critical role for these factors in the process of aneurysm growth (Abdul-Hussien et al.).

Cytokines and Chemokines

Juvonen et al. (1997) measured circulating levels of interleukin-1 beta (IL-1b), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-a), and interferon gamma (IFN-g) in patients with AAA and coronary artery disease (CAD) and in healthy volunteers. IFN-g correlated positively and significantly with aneurysmal expansion rate. Treska et al. (2000) measured TNF-a and IL-8 levels which were found to be significantly low in large and in symptomatic AAA. On the contrary, TNF expression was enhanced in small aneurysms (diameter <50 mm). Additionally, the presence of TNF was mainly observed in aneurysmal walls that were atheromatically changed. In a recent study (Witkowska et al. 2006), TNF expression measured in the serum was significantly higher in AAA patients than in controls, but not related to the aneurysm's size. Local TNF increases in AAA tissue (Newman et al. 1994) might contribute to elevated circulating levels of this cytokine. It should be remembered that TNF production is not only limited to cells of the immune system but also adipose tissue could also be a source of cytokines including TNF. Therefore, TNF, produced by the aneurysmal tissue, might not significantly elevate its serum level. For this reason, implementation of the serum TNF measurements for diagnostic purposes in AAA seems disputable.

C-Reactive Protein

C-reactive protein (CRP) has been shown to be a strong predictor of various cardiovascular events (Ridker et al. 2002). It is a nonspecific acute-phase reactant mainly produced in liver stimulated by various cytokines believed to be involved in the pathogenesis of AAA. Thus it may be a potential biomarker. Norman et al. (2004) found baseline CRP levels being higher in larger aneurysms, but CRP was not associated with expansion rate. Lindholt et al. (2001b) measured CRP in their cohort and could not find any association with size or expansion rate. Serum hsCRP and the size of aneurysm were measured in patients with AAA by Vainas et al. (2005) and showed a modest correlation with aneurysm size. Interestingly, they suggested that CRP produced in vascular tissue might contribute to aneurysm formation. At the same time, Domanovits et al. (2002) compared the level of hsCRP among asymptomatic, symptomatic without rupture, and in patients with rupture of the aneurysm. The presence of symptoms or rupture was significantly correlated with elevated CRP compared to asymptomatic patients. C-reactive protein and D-dimer levels are elevated during early AAA development and CRP levels are also elevated in larger aneurysms but do not appear to be associated with rapid expansion (Norman et al. 2004). The most useful predictor of aneurysmal expansion in men is aortic diameter. There is conflicting evidence about the association between CRP and AAA size or expansion (Norman et al. 2004). In a recent pilot study, a correlation between CRP and AAA size is observed, but without any reliable association with recent AAA growth (Vega de Ceniga et al. 2009). Moreover, it was observed that the association between CRP and AAA size was modulated by statins (Vega de Ceniga et al. 2009).

Haptoglobin (Hp)

Haptoglobin (Hp) is a hemoglobin-binding protein expressed by a genetic polymorphism as three major phenotypes: Hp 1-1, Hp 2-1, and Hp 2-2, originating from two alleles (Hp 1 and Hp 2). Hp 1-1 protein is biologically the most effective in binding free hemoglobin and suppressing inflammatory responses, Hp 2-2 is the least active, and Hp 2-1 is moderately active (Sadrzadeh and Bozorgmehr 2004). The frequency of the Hp 1 allele was significantly increased in patients with aneurysms compared with healthy controls (Sadrzadeh and Bozorgmehr 2004). It has been documented in vitro that Hp 2-1 and Hp 1-1 possess a far higher activity to stimulate elastin hydrolysis by leukocyte elastase than that of Hp 2-2 (Powell et al. 1990). More specifically, Hp 2-2 has no effect on elastin hydrolysis by neutrophil elastase, indicating that Hp 2-1 and Hp 1-1 specifically affect elastin by making it more susceptible to degradation. Corresponding to these data, Hp 2-2 patients had the highest mean age at aneurysm resection (Powell et al. 1990). An increased frequency of the Hp 2-1 phenotype has already been reported among AAA patients (Norrgard et al. 1984), but it is not confirmed in a recent study (Wiernicki et al.). Univariate analysis showed that the Hp 2-1 phenotype was associated with a lower initial AAA diameter, higher AAA growth rate, and shorter time of observation until the last follow-up visit compared with the Hp 1-1 and Hp 2-2 phenotypes. Hp 2-1 was also associated with higher serum elastase activity and CRP concentration than both

homozygous phenotypes (Wiernicki et al.). There were no significant differences in final AAA diameter and neutrophil count, between both Hp 1-1 and Hp 2-2 patients (Wiernicki et al.). AAA growth rate is correlated positively with serum elastase activity and CRP concentration in the entire group as well as in the Hp 2-1 subgroup (Wiernicki et al.). Growth rate is also correlated positively with the neutrophil count in Hp 2-1 patients. No correlations were significant in Hp 1-1 and Hp 2-2 groups. The initial AAA diameter is correlated negatively with serum elastase activity, but this association was significant only in the entire AAA group (Wiernicki et al.). No significant associations between AAA growth rate or inflammation markers and age, gender, smoking, comorbidities, and pharmacologic treatment were found (Wiernicki et al.). More recently, Pan et al. (Pan et al.) found that plasma Hp concentrations were significantly higher in AAA patients (237 $\pm$ 144 vs 163 $\pm$ 86 ng/mL; $p = 0.024$). Further analysis revealed that plasma Hp concentrations were significantly higher in AAA patients with the 2-2 phenotype compared with corresponding non-AAA subjects (238 $\pm$ 144 vs 163 $\pm$ 86 ng/mL; $p = 0.024$).

Selenium (Se)

Clinical evidence collected in numerous studies shown that blood Se drops in inflammatory states (Maehira et al. 2002). The reason for Se reduction is rather obscure and might involve different mechanisms. One concept is related to altered Se distribution in tissues, which most likely contributes to low Se concentration in the circulation (Maehira et al. 2002). Another one is based on excessive turnover of antioxidant enzymes, which contain Se. The results of recent study have shown that although serum Se concentration was lower in the AAA patients than in control group, the observed difference was statistically insignificant (Witkowska et al. 2006). However, when patients were divided depending on the damage to the aorta, serum Se in patients with unruptured aneurysms was similar to that of the controls and patients with ruptured aneurysms had considerably low serum Se concentration (Witkowska et al. 2006). This study also established that the process of the aneurysm's enlargement is related to the reduced Se content in serum (Witkowska et al. 2006). A low serum Se content might indicate an increased demand of the organism for this microelement, which grows along with the growth of the aneurysm (Witkowska et al. 2006).

Table 5 summarizes the published studies reporting the role of circulating biomarkers in the natural history of AAA.

Understanding the molecular mechanisms is an important step toward clarification of the pathophysiology, identification of genetic and molecular biomarkers, and development of new therapeutic strategies for AAA. There are no specific laboratory markers that would allow one to distinguish in a simple way between aneurysm bearers and the healthy population. One or more biomarkers may be indicative for a disease or the risk that a disease will progress, but they are not enough to establish a significant relationship between a biomarker and the aneurysm. Several potential biomarkers for the progression of AAA have been investigated. Serum elastase peptides seem still to be sufficient biomarkers to predict expansion and rupture, but advanced techniques (ELISA) and larger studies are needed to establish its exact role. PAP complexes also may have clinical potential. These biomarkers, whose presence and role in AAA is better established than

Table 5 Summarizes the published studies reporting the role of circulating biomarkers in the natural history of AAA

Biomarker	Author	Findings
CRP	Lindholt (Lindholt et al. 2001b)	CRP did not correlate with size or expansion rate of AAA
	Domanovits (Domanovits et al. 2002)	No significant elevation of CRP in patients who presented symptoms or rupture of an AAA
	Norman (Norman et al. 2004)	CRP levels are elevated in larger aneurysms but do not appear to be associated with rapid expansion
	Vega de Ceniga (Vega de Ceniga et al. 2009)	**C**orrelation between CRP and AAA size without any reliable association with AAA growth. Moreover there is an association between CRP and MA size modulated by statins
hsCRP	Vainas (Vainas et al. 2003)	Serum hsCRP is associated with aneurysmal size
	Parry (Parry et al.)	CRP levels are elevated during early AAA development
D-dimer	Yamazumi (Yamazumi et al. 1998)	The largest diameter of AAA correlated with the preoperative level
	Parry (Parry et al.)	D-dimer levels are elevated during early AAA development
Fibrinogen	Al-Barjas (Al-Barjas et al. 2006)	Fibrinogen may be a useful marker to monitor the progression of AAA
	Yamazumi (Yamazumi et al. 1998)	The largest diameter of MA correlated with the preoperative of FDP
PIIINP	Satta (Satta et al. 1997)	Acceleration of AAA growth is reflected in serum PIIINP
	Treska (Treska and Topolcan 2000)	The plasma level of PIIINP cannot be used as marker
	Lindholt (Lindholt et al. 2000)	A predictive model using PIIINP and initial of predicting nine out to ten AAAs that will be operated on within 5 years
	Wilson (Wilson et al. 2001)	Increased elastolysis is associated with in increased AAA wall distensibility; over is associated with distensibility
Elastase	Lindholt (Lindholt et al. 2003a)	P-elastase was positively correlated with the mean annual AAA expansion rate
Cystatin C	Shi (Shi et al. 1999)	Increased abdominal aortic diameter correlated inversely with serum cystatin C levels
	Lindholt (Lindholt et al. 2001a)	Deficiency of cystatin C was associated with increased aneurysm size and expansion rate
MMPs	Lindholt (Lindholt et al. 2000)	Plasma MMP-9 may predict the natural history of AAA
	Eugster (Eugster et al. 2005)	Both MMP-2 and -9 and NIIINP failed to show relevance as serum markers for aortic dilatation
	Sangiorgi (Sangiorgi et al. 2001)	MMPs may indicate successful AAA exclusion after endovascular repair while persistently high levels may indicate an endoleak

(*continued*)

Table 5 (continued)

Biomarker	Author	Findings
	Hovsepian (Hovsepian et al. 2000)	MMP-9 concentrations were higher in patients with AAA compared with patients with aortic occlusive disease or healthy subjects
	Wilson (Wilson et al. 2008a)	MMP-9 and MMP-1 were significantly elevated in the plasma of ruptured AAA compared with non-ruptured AAA
	Speelman (Speelman et al.)	Only MMP-9 showed a positive correlation
tPA	Lindholt (Lindholt et al. 2003b)	AAA progression may be partly caused by an activation of plasminogen by tPA
	Reilly (Reilly et al. 1994)	Higher levels of tPA in aneurysmal aortas compared with normal aortas and occlusive aortas
TNF-a, IL-8	Treska (Treska et al. 2000)	IL-8 and TNF-alpha can be used as endogenous markers of the process of AAA development
	Witkowska (Witkowska et al. 2006)	TNF expression was higher in AAA patients than in controls, but not related to the aneurysm's size
IL-6	Rodhe (Rohde et al. 1999)	IL-6 was independently correlated with indexed aortic diameter
	Jones (Jones et al. 2001)	Circulating levels of IL-6 were significantly higher in patients with AAAs than in controls
	Dawson (Dawson et al. 2006)	IL-6 may play a role in the early biologic processes of aortic dilatation that eventually leads to aneurysm development
	Dawson J (Dawson et al. 2007)	Larger-volume aneurysm provides larger surface area, leading to higher concentrations of IL-6
PAP	Lindholt (Lindholt et al. 2001a)	The progression of AAA is correlated with the PAP level
INF-g	Juvonen (Juvonen et al. 1997)	INF-gamma concentrations seem to predict an increased rate of expansion in AAA
MIF	Pan (Pan et al. 2003)	An association between serum MIF level and AAA initial size and AAA expansion rate
Se	Witkowska (Witkowska et al. 2006)	Se concentration was lower in the AAA patients than in the control group
Lp (a)	Takagi (Takagi et al. 2009b)	Circulating Lp (a) concentrations may be higher in patients with AAA than those in subjects without AAA
	Giusti (Giusti et al. 2009)	Association between decreased expression levels of LRP5 gene and increased levels of Lp(a) in AAA patients
Hp	Wiernicki (Wiernicki et al.)	Hp 2-1 phenotype was associated with a lower initial AAA diameter and higher AAA growth rate compared with the Hp 1-1 and Hp 2-2 phenotypes
TIMP-1	Nakamura (Nakamura et al. 2000)	Plasma concentrations of TIMP-1 were found to be significantly higher in AAAs than in healthy controls
	Wilson (Wilson et al. 2006)	TIMP-1 levels were found to be lower in AAA wall tissue compared to healthy aortic tissue

(*continued*)

Table 5 (continued)

Biomarker	Author	Findings
al-AT	Vega de Ceniga (Vega de Ceniga et al. 2014)	A1-AT is correlated with recent AAA growth
	Takagi (Takagi et al. 2009c)	A1-AT has been associated with the future development of AAA and has been correlated with AAA expansion but not size

the novel ones, seem to be correlated also with ILT, where thin ILT is found to be an independent predictor of high MMP-9 and CRP concentrations (Wiernicki et al.).

Novel Biomarkers and Its Potential Role in AAA

Biomarkers Related to Extracellular Matrix Homeostasis or Proteolysis

Cystatin C

Cystatin C is the endogenous inhibitor of the elastolytic enzymes cathepsins, which are strongly expressed in the aneurysmatic wall. Cystatin C expression is found reduced in AAA disease, leading to lack of inhibition of the elastolytic properties of cathepsins. In a prospective study (Lindholt et al. 2001a), a negative correlation of serum cystatin C values with AAA size and annual expansion rate was found, but without mentionable potential for predicting cases requiring surgery.

Circulating Basement-Membrane (BM) Fragments

Type IV and XVIII of collagen are components of the basement membrane. Ramazani et al. (Ramazani et al.) have recently shown that AAA patients had significantly increased levels of type IV and XVIII collagen compared with the controls ($p = 0.005$ and $p < 0.001$, respectively). Moreover, AAA patients had significantly increased level of type XVIII collagen ($p < 0.01$) when compared with the peripheral arterial disease (PAD) group (Ramazani et al.).

This study was conducted in a small number of patients, indicating that further studies are required to establish the potential role of BM fragments as biomarkers for AAA.

Osteoprotegerin (OPG)

It has been suggested that serum osteoprotegerin (OPG) levels are associated with growth of AAAs, while in vitro experiments showed that OPG promotes matrix metalloproteinase (MMP) release from monocytes and vascular smooth muscle cells. In a recent study (Koole et al. 2012), the concentration of aortic wall OPG was positively associated with established markers of AAA severity (cathepsins A, B, and S and the activity of MMP-2 and MMP-9), while it appeared to be associated with lymphocytes and plasma cells. These newer data in humans suggest a role for OPG in AAA pathogenesis.

Biomarkers Related to Cellular Signaling Pathways

Diminished Soluble Tumor Necrosis Factor-Like Weak Inducer of Apoptosis (sTWEAK)

sTWEAK is a type II transmembrane glycoprotein of the TNF superfamily that circulates in plasma (Chicheportiche et al. 1997) and is expressed in SMCs and leukocytes in arterial wall. TWEAK was found reduced in patients with coronary artery disease, carotid atherosclerosis, or PAD (Moreno et al.). Martín-Ventura et al. (Martin-Ventura et al.) measured sTWEAK plasma levels in patients with AAA and found that sTWEAK concentrations were decreased in small ($\leq$5 cm, $p = 0.03$) as well as large AAA (>5 cm, $p = 0.004$) compared with healthy subjects. Moreover, sTWEAK concentrations were negatively associated with AAA size ($p = 0.008$) and AAA expansion rate with 5 years of follow-up ($p = 0.031$) (Martin-Ventura et al.).

These results show that sTWEAK is strongly associated with the presence of an aneurysm, but fails to differentiate among small and large aneurysms (Martin-Ventura et al.).

Tenascin-C (TN-C)

TN-C is a matricellular (extracellular matrix) protein that is synthesized by various cell types including vascular smooth muscle cells (VSMC) in response to inflammatory cytokines and mechanical stress (Mackie et al. 1992). TN-C is typically synthesized in pathological conditions like wounds, inflammation, and tumorigenesis (Midwood and Orend 2009). Such a biomarker could be useful in stratifying risk in patients with AAA before and after EVAR intervention, since in some patients aneurysms continue to grow even after the successful deployment of the stent graft, possibly because of continuing inflammation (Greenhalgh et al.). Furthermore, no biomarker is available for indicating the pathological status of VSMC and interstitial cells, and TN-C may be useful for this purpose, possibly in combination with other inflammatory markers and ECM degradation products. TN-C has the advantage of being deposited locally in the inflammatory lesion (AAA) and it is also released in stable forms into circulation (Kimura et al.).

Further studies are required to elucidate the complete function of TN-C and to evaluate whether serum levels or bio-imaging of TN-C is better suited for the assessment of disease activity in human AAA.

Proteins Released by Intraluminal Thrombus (ILT)

Peroxiredoxin-1 (PRX-1)

Martinez-Pinna et al. (Martinez-Pinna et al.) analyzed proteins released by intraluminal thrombus (ILT) with proteomic approach and found that PRX-1 was more released by the luminal layer compared with the abluminal layer of the ILT. Increased PRX-1 serum levels in AAA patients compared with healthy subjects and a positive correlation among PRX-1 and AAA diameter and expansion rate were also

found. The combination of PRX-1 and AAA size seems to be significantly predictive of AAA growth (Martinez-Pinna et al.), establishing PRX-1 as a promising biomarker.

Neutrophil Gelatinase-Associated Lipocalin (NGAL)

NGAL plasma concentrations have been associated with cardiovascular diseases (Prabhu et al.). Polymorphonuclear cells (PMNs) isolated from AAA patients secreted significantly greater amounts of NGAL, than PMNs from controls and correlated with retrospective AAA growth (Ramos-Mozo et al.). The ILT releases large amounts of NGAL compared to the abluminal thrombus, the aneurysm wall, and the healthy aortic media. Further studies in larger subjects groups are needed to confirm the association between NGAL and AAA presence and growth (Ramos-Mozo et al.).

It has been suggested that the ILT of AAAs predisposes for enlargement and rupture. The growth of the AAA is dependent on proteolytic degradation of elastin. NGAL can bind to MMP-9 and inhibit its degradation, thereby preserving enzymatic activity (Folkesson et al. 2007). Complexes of NGAL and active MMP-9 were present in the thrombus, the interface fluid, and the aneurysm wall. Still, the importance of these observations is unknown and the contribution of the complex NGAL/MMP-9 to the AAA growth should be further evaluated (Folkesson et al. 2007).

Insulin-Like Growth Factors and Its Binding Proteins (IGFs and IGFBPs)

Lindholt et al. (Lindholt et al.) have evaluated the potential role of IGF-I and IGF-II as biomarkers in 115 patients with AAA, kept under annual surveillance for 10 years. Serum IGF-I correlated positively with AAA size and growth rate ($p = 0.016$ and $p = 0.004$, respectively), findings that persist after adjustment for potential confounders. Serum IGF-I level predicted cases needing later surgery (95 % confidence interval(CI):0.52–0.73) (Lindholt et al.).

IGFBP-1 was localized in the luminal part of AAA thrombus and IGFBP-1 levels were increased in AAA thrombus-conditioned media, compared to media layer and healthy media (Ramos-Mozo et al.). It seemed to facilitate the potentiation of ADP-induced platelet aggregation triggered by IGF-1, while its concentrations were significantly higher in large AAA patients compared with control subjects (normal aortic size) ($p < 0.01$). Moreover, IGFBP-1 levels correlated with AAA size ($p < 0.001$), which remained significant after adjusting for risk factors (Ramos-Mozo et al.).

Biomarkers Related to Circulating Cells and Inflammation

Lymphocytes

Increasing evidence shows that the autoimmune response contributes importantly to the pathogenesis of AAA. More specifically, CD4(+), CD25(+), and FOXP3(+) T regulatory cells (Tregs) were found significantly decreased in AAA patients compared to the control group ($p < 0.01$), indicating impaired immunoregulation (Yin et al.).

Additionally, the loss of the inhibitory receptor CD31 on peripheral T lymphocytes is found to be associated with the incidence of atherosclerotic complications such as AAA in patients (Fornasa et al.). These findings should be further researched in order to establish potential biomarkers of the natural history of AAA, as well as potential therapeutic targets for the inhibition of the creation and progression of aneurysms.

In another study, increased plasma levels of sCD28 and sCD86 ($p = 0.0001$) and decreased plasma levels of sCTLA-4 ($p = 0.0018$) were found in AAA patients compared with normal individuals. These levels were not related to the patient's age or the size of aneurysm, but there was a significant inverse relationship between the concentrations of sCTLA-4 and sCD80 with matrix metalloproteinase-9 (Sakthivel et al. 2007).

Monocytes

In a more recent study (Ghigliotti et al. 2013), CD16(+) monocyte subsets were found increased in large abdominal aortic aneurysms and were differentially related with circulating and cell-associated (CD143) biochemical and inflammatory biomarkers. A clinical implication of this study is that by taking common blood measurements (plasma D-dimer, creatinine, and age to derive eGFR, uric acid, total white blood, and neutrophil counts), one could discriminate AAA patients with different monocyte-dependent inflammatory profiles. This study was hampered by cross-sectional design and the relatively small number of patients, while it was unable to find a clear relation of the size of AAA and the values of circulating monocyte subsets in patients at different stages of expansion of aortic damage.

Progenitor Cells

CD34(+) levels are a known marker of circulating progenitor cells. Van Spyk et al. (2013) investigated the role and compared the percentage of CD34(+) cells in AAA disease and peripheral vascular disease (PVD). This small study revealed a lower percentage of CD34(+) cells in AAA patients, compared to PVD patients, concluding that AAA is a less severe vascular disease than PVD. Further study is needed in order to establish CD34(+) cells as a biomarker for risk stratification.

Lymphangiogenesis

While angiogenesis is a known factor in the inflammatory environment of AAAs and lymphangiogenesis has been associated with chronic inflammatory conditions, it has not yet been associated with AAA. A study by Scott et al. (2013) attempted to research the relationship between inflammation and neovascularization in AAA tissue. The results showed that the aneurysm wall contained high levels of inflammatory infiltrate, while microvascular densities of blood ($P < 0.001$) and lymphatic ($P = 0.003$) vessels were significantly increased in AAA samples compared with controls. Vascularity correlated positively with inflammation, while increased VEGFR-3 and VEGF-A expression was observed within inflammatory areas of AAAs. These results suggest lymphatic vessel involvement in AAA disease, associated with the extent of inflammation (Scott et al. 2013).

Catalase

PMNs play a key role in AAA progression. Diminished catalase expression and activity were observed in PMNs from AAA patients compared with controls. Catalase plasma levels were also decreased in large and small AAAs when compared with healthy individuals. This study was also conducted in a very small number of patients (Ramos-Mozo et al.).

Metabolomics

Metabolomics stand for sensitive analytical techniques such as metabolic fingerprinting with multivariate analysis. Metabolomics seems to be a good approach to find biomarkers of AAA (Ciborowski et al.).

Guanidinosuccinic Acid (GSA)

Guanidinosuccinic acid (GSA), which is mainly released from the ILT, is highly increased in the plasma of AAA patients when compared to controls. GSA behaves like nitric oxide (NO), due to its vasodilatory actions and its ability to activate the NO generating N-methyl-D-aspartate (NMDA) receptor. After being generated in the ILT, GSA is secreted to blood stream, and the amount of secreted GSA is related to the stage of AAA (Aoyagi et al. 2001).

Hippuric acid

Hippuric acid is secreted only by the luminal part of the ILT and was found significantly decreased in the plasma of AAA patients. This observation correlates with the hyperexcretion of hippuric acid in atherosclerotic state (Ciborowski et al.).

Long-Chain Acylcarnitines

Long-chain acylcarnitines were decreased in the plasma of AAA patients compared to controls. There was a clear decreasing trend with increasing size of aneurysm which may indicate altered fatty acid β-oxidation or deficiency of carnitine (Ciborowski et al.). Additionally, a significant decrease in sphingosine 1-phosphate (S1P) and sphinganine-1-phosphate in AAA patients was also found. Both molecules are sphingolipids; sphinganine-1-phosphate is a parent of sphingosine and S1P. S1P is a lysophospholipid, and significant changes in other lysophospholipids like lysoPEs and lysoPCs are reported in this investigation. A possible explanation for decrease in the amount of lysoPCs/PEs in plasma of AAA patients, with a trend related to the size of aneurysm, is their accumulation in the ILT (Ciborowski et al.).

Vitamin D-Binding Protein (DBP) and Vitamin D

During the last decades there has been a surge of interest in vitamin D and its wide range of health benefits, partially due to the many association studies linking vitamin D status with common human diseases. DBP is the main serum carrier of vitamin D metabolites, with albumin acting as an alternative lower affinity binder (Bikle

et al. 1986). Gamberi et al. (Gamberi et al.) showed a negative correlation between DBP and the presence of AAA. Even if its value is not well established yet, DBP is pivotal for vascular remodeling and it may have an important role in the protection of vascular walls.

In a more recent observational study (Wong et al. 2013a), 4233 older men (70–88 years old) participated in a randomized controlled trial of screening for AAA. The study measured their infrarenal aortic diameter by ultrasound and their 25(OH)D plasma levels by immunoassay. An inverse relationship between vitamin D status and the presence of larger AAA was found, along with an inverse dose–response association between 25(OH)D concentrations and the size of AAA, suggesting a role of vitamin D in the severity of aneurysmal arterial disease. Further research is needed to clarify the mechanisms underlying these associations.

Homocysteine

Wong et al. (Wong et al. 2013b) investigated in a cross-sectional study the relationship of homocysteine and the presence and diameter of AAAs in older men (70–88 years old). Plasma total homocysteine (tHcy) was found to be associated with the presence of AAA, while there was also a positive dose–response relationship between tHcy and abdominal aortic diameter. The investigators concluded that further, longitudinal studies and clinical trials of lowering tHcy are required in order to assess if these relationships are causal.

Lipoproteins and Lipoprotein-Related Receptors

Results of a meta-analysis suggest that circulating lipoprotein alpha (Lp(a)) concentrations may be higher in patients with AAA than those in subjects without AAA, thus playing a role in the diagnosis of AAA (Takagi et al. 2009b). Low-density lipoprotein receptor-related protein-1 (LRP1) demonstrated significant association with AAA size ($p = 0.0042$) (Bown et al.). In a small pilot study in 12 patients by Chan et al. (2013), lipoprotein receptor-related protein-1 (LRP1) expression was found significantly lower in AAA patients than controls, while no significant correlation was shown between LRP1 protein expression and the size of AAA ($p > 0.05$). These results suggest that a reduction in LRP1 protein expression could be associated with aneurysm progression.

Other metabolites used to discriminate the natural history of patients with large aneurysm, small aneurysm, and controls are the metabolites of cholesterol. Decreased plasma levels of those metabolites were observed in patients with large and small aneurysms in comparison to controls (Ciborowski et al.). Lower serum HDL cholesterol and higher serum LDL cholesterol may be associated with the presence of AAA (Takagi et al.). The serum HDL concentrations were lower in patients with AAAs and were independently associated with a reduced risk of having an AAA, in men not receiving current lipid-modifying therapy (95 % CI 0.56–0.93 per 0.4-mM increase) and in the total cohort (95 % CI 0.63–0.91 per 0.4-mM increase). The concentrations of LDL and triglycerides were not associated with the presence of AAAs (Golledge et al.).

Past studies have revealed decreased low-density lipoprotein receptor-related protein-5 (LRP5) gene expression in peripheral blood cells of AAA patients and an association between decreased expression of LRP5 and increased lipoprotein (a) (Lp(a)) levels in AAA patients. In a recent study (Galora et al. 2013), LRP5 gene polymorphisms rs4988300 and rs3781590 were found independent genetic markers of AAA, even after adjusting for age, sex, dyslipidemia, hypertension, smoking habit, and chronic obstructive pulmonary disease. AAA patients had significantly higher Lp(a) levels than control subjects ($P < 0.0001$). Further studying of the role of these markers in AAA and of LRP5 gene in Lp(a) catabolism and AAA pathophysiology is necessary.

Phospholipases

Wallinder et al. (Wallinder et al.) showed that patients with small AAAs had increased levels of the enzyme glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD) compared with controls without aneurysm by using a proteomic approach, providing some evidence of the value of GPI-PLD as a novel potential plasma biomarker for the detection of AAAs (Wallinder et al.). In the same tone, Golledge et al. found (Golledge et al.) that serum secretory phospholipase A (2) (sPLA(2)) activity is elevated in men with small AAAs but is not associated with AAA progression.

Genetic Biomarkers

Telomere Length

Telomeres are specialized DNA sequences at the ends of linear chromosomes. Telomere attrition is the phenomenon of telomere length "shortening" with each successive cell division, eventually leading to cell senescence and/or apoptosis. These observations can contribute to the estimation of the cellular biological age (Wilson et al. 2008b). In humans, reduced telomere length in circulating leukocytes is associated with premature vascular disease, with the telomere/genomic DNA content being significantly reduced in wall biopsies of AAA compared to normal aortas ($p < 0.001$) (Wilson et al. 2008b). This decreased telomerase endothelial expression implies a protective role of telomerase against AAA formation (Dimitroulis et al.). Lowest telomere restriction fragment (TRF) length values double the risk of having AAA compared with a mean TRF length in the highest values ($p = 0.005$) (Atturu et al.).

AAA1 Locus on Chromosome 19q13

Several single nucleotide polymorphisms (SNPs) were nominally associated with AAAs ($p < 0.05$) (Lillvis et al.). The SNPs with most significant p-values were peptidase D (PEPD) and CD22 (Lillvis et al.). Immunohistochemical staining for CD22 revealed protein expression in lymphocytes present in the aneurysmal aortic wall only and no detectable expression in control aortas (Lillvis et al.). PEPD protein

was expressed in fibroblasts and myofibroblasts in the media-adventitia border in both aneurysmal and non-aneurysmal tissue samples (Lillvis et al.).

9p21

AAA is among a number of vascular disorders to be recently associated with a common allelic variant situated on chromosome 9p21 (Thompson et al. 2009). A significant association between rs10757278-G and the presence of AAA was found ($p = 0.03$), an effect size completely consistent with that originally reported (Thompson et al. 2009). rs10757278 was not significantly associated with altered AAA growth rate (Thompson et al. 2009). In a newer study (Wei et al. 2013), single nucleotide polymorphisms rs10757278 and rs1333049 of chromosome 9p21-3 region were significantly associated with increased risk of AAA. This study revealed no association between polymorphisms and aortic diameters in AAA patients, while a specific genotype (GG of rs10757278) was suggested to interact with the homocysteine biological pathway to stimulate the presence of AAA. This data emphasized the need to further study the role of these biomarkers.

Chemokine Receptors (CCRs)

Chronic inflammation plays an important role in AAA formation. The chemokine receptors CCR2, CCR5, and CXCR3 are associated with pathways implicated previously in aneurysm pathogenesis. Chemokine receptor-2 (CCR2) is involved in the regulation of the inflammatory response (Katrancioglu et al.). CCR2 heterozygote V64I polymorphism and allele frequency were more frequently observed in the AAA group ($p = 0.01$, $p = 0.004$) (Katrancioglu et al.), but there was no significant difference with the control group in relation to the Delta32 allele frequency (Sandford et al. 2009).

Table 6 summarizes the published studies on novel biomarkers in AAA and their clinical value.

In the second part of the chapter, the aim is to give a description of the emerging biomarkers that can correlate with the existence (presence/diagnosis) and progression (size/risk of rupture) of abdominal aortic aneurysms (AAAs). The clinical value of biomarkers is based on their properties to satisfy the goals of early detection (screening), surveillance in terms of early and later progression, and in monitoring the biologic performance of an AAA after surgical treatment. An ideal biomarker should be able to be applied in all of the above. Early detection and close surveillance can greatly benefit patients, since they are referred for elective repair with a lower risk, compared with the emergency setting (Greenhalgh et al.; Harris et al. 2006). The mortality of elective AAA repair has been 5 % or less, but once rupture occurs, operative mortality is as high as 48 %.

As presented, numerous biomarkers, related to the AAA disease, are currently being researched. Most of these studies are either experimental or hampered by their low numbers of patients.

sTWEAK is strongly correlated with aneurysm existence or expansion rate but fails to differentiate small to large aneurysms. TN-C contributes in stratifying risk in

Table 6 Summarizes the published studies on novel biomarkers in AAA and their clinical value

Category	Biomarker	Author	Year	Study design	Findings	Clinical value
Biomarkers related to cellular signaling pathways	CRP	De Haro (De Haro et al. 2012)	2012	Cohort study	A statistical association was confirmed between the AAA diameter and high-sensitivity CRP (hsCRP) plasma levels	Aneurysm progression
	sTWEAK	Martín-Ventura (Martin-Ventura et al.)	2010	Case control study	sTWEAK plasma levels in patients with AAA compared with healthy subjects. sTWEAK concentrations were negatively associated with AAA size and AAA expansion rate	Aneurysm detection and progression
	Tenascin-C (TN-C)	Greenhalgh (Greenhalgh et al.)	2010	Follow-up study	TN-C is used to stratify risk in patients with AAA before intervention and also after endovascular repair	Aneurysm progression and treatment efficacy
Biomarkers related to circulating cells and inflammation	Lymphocyte correlated biomarkers	Yin (Yin et al.)	2010	Prospective study	CD4(+)CD25(+)FOXP3(+) T cells in AAA patients are found significantly lower than that of the control group	Aneurysm detection
	Monocyte-related biomarkers	Ghigliotti (Ghigliotti et al. 2013)	2013	Case control study	CD16(+) monocyte subsets were found increased in large abdominal aortic aneurysms and were differentially related with circulating and cell-associated (CD143) biochemical and inflammatory biomarkers	Aneurysm size
	Progenitor Cells	Van Spyk (Van Spyk et al. 2013)	2013	Case control study	A lower percentage of CD34(+) cells in AAA patients, compared to PVD patients, was found	Aneurysm detection

	Lymphangiogenesis	Scott (Scott et al. 2013)	2013	Case control study	Lymphatic vessel involvement in AAA disease, associated with the extent of inflammation	Aneurysm genesis
	Catalase (PMNs)	Ramos-Mozo (Ramos-Mozo et al.)	2011	Case control study	Diminished catalase expression and activity were observed in PMNs from AAA patients compared with controls	Aneurysm detection
Proteins released by intraluminal thrombus (ILT)	IGFs	Lindholt (Lindholt et al.)	2011	Prospective study	Serum IGF-I correlated positively with AAA size and growth rate	Aneurysm size and progression
		Ramos-Mozo (Ramos-Mozo et al.)	2012	Case control study	IGFBP-1 concentrations were significantly higher in large AAA patients compared with control subjects	Aneurysm size and thrombus existence
	NGAL	Ramos-Mozo (Ramos-Mozo et al.)	2011	Case control study	AAA patients secreted significantly greater amounts of NGAL than PMNs from controls and correlated with retrospective AAA growth	Aneurysm detection and progression
	Peroxiredoxin-1	Martinez-Pinna (Martinez-Pinna et al.)	2011	Prospective study	Increased PRX-1 serum levels in AAA patients compared with healthy subjects and positive correlation among PRX-1 and AAA diameter and expansion rate was also found	Aneurysm detection and progression
Biomarkers related to extracellular matrix homeostasis or proteolysis	Cystatin C	Lindholt (Lindholt et al. 2001a)	2001	Prospective study	Negative correlation with aneurysm size and expansion, no potential for predicting surgery	Aneurysm size and progression
	Circulating basement-membrane fragments	Ramazani (Ramazani et al.)	2011	Case control study	AAA patients had significantly increased levels of type IV and XVIII collagen compared with the controls	Aneurysm detection

(*continued*)

Table 6 (continued)

Category	Biomarker	Author	Year	Study design	Findings	Clinical value
	OPG	Koole (Koole et al. 2012)	2012	Retrospective study	Aortic wall OPG was positively associated with established markers of AAA severity, while it appeared to be associated with lymphocytes and plasma cells	Aneurysm genesis and growth
Genomic Biomarkers	Telomere length	Wilson (Wilson et al. 2008b)	2008	Case control study	Telomere/genomic DNA content being significantly reduced in wall biopsies of AAA compared to normal aortas	Aneurysm detection
	AAA1 locus on chromosome 19q13	Lillvis (Lillvis et al.)	2011	GWAS	CD22 revealed protein expression in lymphocytes present in the aneurysmal aortic wall only and not in control aorta. PEPD protein was expressed in fibroblasts and myofibroblasts in the media-adventitia border in both aneurysmal and non-aneurysmal tissue samples	Aneurysm detection
	9p21	Thompson (Thompson et al. 2009)	2009	Cohort study	A significant association between rs10757278-G and the presence of AAA was found ($p = 0.030$). rs10757278 was not significantly associated with altered AAA growth rate	Aneurysm detection
		Wei (2013)	2013	Case control study	SNP rs10757278 and rs1333049 of chromosome 9p21-3 region were significantly associated with increased risk of AAA. This study revealed no association between polymorphisms and aortic diameters in AAA patients	Aneurysm genesis

	CCRs	Katrancioglu (Katrancioglu et al.)	2011	Case control study	CCR2 heterozygote V64I polymorphism and allele frequency were more frequently observed in the AAA group	Aneurysm detection
Metabolomics	Lps	Takagi (2009b)	2009	Meta-analysis	Lp(a) concentrations may be higher in patients with AAA than those in subjects without AAA	Aneurysm detection
		Takagi (Takagi et al.)	2010	Meta-analysis	Lower serum HDL cholesterol and higher serum LDL cholesterol may be associated with AAA presence	Aneurysm detection
		Chan (2013)	2013	Case control study	Lipoprotein receptor-related protein-1 (LRP1) expression was found significantly lower in AAA patients than controls, while no significant correlation was shown between LRP1 protein expression and the size of AAA ($p > 0.05$)	Aneurysm detection
		Giusti (2009)	2009	Case control study	Association between decreased expression levels of LRP5 gene in AAA patients	Aneurysm detection
		Galora (2013)	2013	Case control study	LRP5 gene polymorphisms rs4988300 and rs3781590 were found independent genetic markers of AAA. AAA patients had significantly higher Lp(a) levels than control subjects ($P < 0.0001$)	Aneurysm detection
	Vitamin D-binding protein	Gamberi (Gamberi et al.)	2011	Proteomics	Negative correlation between DBP and AAA presence	Aneurysm detection

(*continued*)

Table 6 (continued)

Category	Biomarker	Author	Year	Study design	Findings	Clinical value
		Wong (Wong et al. 2013a)	2013	Randomized clinical study	An inverse relationship between vitamin D status and the presence of larger AAA was found, along with an inverse dose–response association between 25(OH)D concentrations and the size of AAA	Aneurysm size
	Homocysteine	Wong (Wong et al. 2013b)	2013	Cross-sectional study	Plasma total homocysteine (tHcy) was found to be associated with the presence of AAA, while there was also a positive dose–response relationship between tHcy and abdominal aortic diameter	Aneurysm detection and size
	Phospholipases	Wallinder (Wallinder et al.)	2012	Case control study	Small AAA had increased levels of the enzyme glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD) compared with the controls without aneurysm	Aneurysm size and detection

patients with AAA before intervention or after EVAR (Greenhalgh et al.), but further study is required to elucidate the function of TN-C and to evaluate whether serum levels or bio-imaging of TN-C would be suited for the assessment of disease activity in human AAAs.

As far as biomarkers related to ILT is concerned, IGF and PRX-1 seem to be the most promising due to their statistically established correlation with AAA diameter and growth (Martinez-Pinna et al.),.

Genetic factors did not offer statistically significant results, even though it remains a wide and undiscovered research field especially as far as the application of chromosome 12 loci in AAA, not just in cases of aortic dissection (Pan et al.).

As far as metabolomics is concerned, they are probably beneficial in terms of early detection of AAA, but it is not clear in literature how they are affected and in turn biased from the metabolic status of AAA patients (many of them are smokers or suffer from hypertension or/and dyslipidemia, which are risk factors for developing AAA). Additionally, they present the tendency to increase the percentage of change and significance with the size of aneurysm. Therefore, identified metabolites could be good targets for the early detection of AAA.

However, none of the aforementioned biomarkers can adequately present the combination of all the pathophysiological events that generate and expand an AAA. This means that there is not a biomarker simultaneously indicative for ILT presence, inflammation, and proteolysis.

Biomarkers may help to explain pathological processes of AAA existence and expansion and allow us to find novel therapeutic strategies or to determine the efficiency of current therapies. To date, there are no specific laboratory markers, which allow us to screen for the disease and monitor its progression or the results of treatment. Further studies and studies in larger patient groups are required in order to validate biomarkers as cost-effective tools in the AAA disease. Advances in modern science guide medicine toward minimally or noninvasive techniques for the diagnosis and management of diseases. Surgery and in particular abdominal aortic aneurysms are no exception and less invasive techniques, like EVAR, are already gaining ground, compared to older methods. This technological progress will hopefully make biomarkers a reality for the screening, monitoring, and choosing the optimal time of intervention in abdominal aneurysms.

Potential Applications to Prognosis, Other Diseases, or Conditions

The most feared complication of AAA disease is rupture, which often leads to the patient's death. So, the earlier the diagnosis through screening is set, the more likely is to offer the appropriate management.

Surgical correction remains the only effective and "finite" treatment of AAA, with the optimal timing for surgery being the main debatable point.

Elective repair has a better overall outcome when repair of AAA is performed on elective basis compared to the emergency repair.

Traditional AAA screening, evaluation, and surveillance programs employ the use of imaging techniques such as CT angiogram (CTA), ultrasound (sonography) (US), or magnetic resonance imaging (MRI). Despite the proven efficacy of these imaging techniques, the cost associated with such programs can incur significant financial burdens to the health care systems, so alternative methods are continuously being researched, with the most discussed in the literature, that of biomarkers.

In order for a biomarker to be useful, it should be able to either detect the disease itself or express its progression. So a useful biomarker should detect the presence of a subclinical aneurysm or be a measure of its size and expansion rate, thus predicting the risk of rupture.

Furthermore a biomarker could define the optimal surveillance intervals and possibly identify pathogenic pathways which could guide monitoring and treatment. We should not fail to mention that in order for a biomarker to be employed in modern healthcare system, its use should be cost-effective. Some of the patients selected through this process should be pointed toward more focused screening by specialized imaging techniques. Circulating biomarkers thus present as attractive alternatives for screening and monitoring purposes particularly in healthcare systems which lack the infrastructure to support other primary or secondary screening programs.

Summary Points

- Abdominal aorta aneurysm (AAA) is a serious threat for human life, especially in such cases when it is asymptomatic until aneurysm rupture, which is a general cause of death in AAA subjects.
- This chapter focuses on a twofold objective: to give a conceptual description of the potential biomarkers that can correlate and predict the natural history of an AAA and to summarize the developments in the literature concerning the novel biomarkers and their potential screening and therapeutic values.
- The first part of the chapter describes the biomarkers which are correlated with plasmin, plasmin activators, and the fibrinolytic system and with inflammation interactions with AAA pathogenesis.
- The second part of the chapter summarizes the following categories of novel biomarkers: biomarkers related to extracellular matrix homeostasis or proteolysis, biomarkers related to cellular signaling pathways, proteins released by intraluminal thrombus (ILT), biomarkers related to circulating cells and inflammation, metabolomics, and genetic biomarkers.
- In conclusion, there are no specific laboratory markers that would allow one to distinguish in a simple way between aneurysm bearers and the healthy population.
- Future research is required in order to establish the underlying relations between the referred biomarkers and their role in AAA pathophysiology.

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Cardiac Biomarkers in Cirrhosis and Portal Hypertension: Relation to Circulatory and Cardiac Dysfunction

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_19

Abstract

Liver cirrhosis is a chronic disease often characterized by pronounced hemodynamic alterations affecting both the systemic and the splanchnic circulation. These circulatory changes lead to the development of extrahepatic complications involving numerous organ systems as a multi-organ failure syndrome. In the heart, alterations of the cardiac function are frequently reported with a decrease in cardiac performance and the systolic and diastolic function, which may contribute to other complications such as renal failure. The circulatory changes activate potent counter-regulatory neurohumoral mechanisms including the renin-angiotensin-aldosterone, the sympatho-adrenergic, and the vasopressin systems. Additionally, several vasoactive substances are involved in the pathogenesis of the circulatory and cardiac dysfunction in cirrhosis, and within the recent years our knowledge on the role of natriuretic peptides, cardiac troponin, endothelins, calcitonin gene-related peptide, adrenomedullin, and nitric oxide has improved considerably. Furthermore, activated cytokines such as IL-6 and TNF-α seem to aggravate the circulatory dysfunction. Cardiovascular markers have been related to both morbidity and mortality in advanced cirrhosis and may therefore serve as important prognostic markers.

Keywords

Cirrhosis • Chronic liver disease • Portal hypertension • Cirrhotic cardiomyopathy • Hyperdynamic circulation • Cardiac dysfunction • Vasoactive markers

Abbreviations

ANP	Atrial natriuretic peptide
AVP	Arginine vasopressin (also known as the antidiuretic hormone)
BNP	Brain natriuretic peptide
CGRP	Calcitonin gene-related peptide
CNP	C-type natriuretic peptide
HPS	Hepatopulmonary syndrome
HRS	Hepatorenal syndrome
Hs-TnT	High-sensitivity troponin T
IL-6	Interleukin 6
LVEF	Left ventricle ejection fraction
NO	Nitric oxide
ProADM	Adrenomedullin prohormone
ProANP	Atrial natriuretic peptide prohormone
ProBNP	Brain natriuretic peptide prohormone
ProCNP	C-type natriuretic peptide prohormone
RAAS	Renin-angiotensin-aldosterone system

SBP	Spontaneous bacterial peritonitis
SNS	Sympathetic nervous system
TIPS	Transjugular intrahepatic portosystemic shunt
TNF-α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor

Key Facts of Cirrhosis and Portal Hypertension

- Cirrhosis is a chronic liver disease primarily caused by viral hepatitis or excessive alcohol intake.
- The liver becomes filled with scar tissue and therefore the portal pressure increases.
- The function of circulation and the heart may become impaired.
- The patients may develop complications such as water retention in the abdomen, coma, and renal failure.
- Various hormones and substances are released in the circulation and contribute to the development of complications.
- The only curable treatment is liver transplantation.

Definitions

Ascites Ascites constitutes the formation of fluid in the abdominal cavity. It is a common complication in cirrhosis occurring due to the profound circulatory changes in these patients.

Bacterial translocation Bacterial translocation is defined as an increased intestinal permeability that facilitates the movement of bacteria from the gastrointestinal tract to mesenterial lymph nodes in patients with cirrhosis.

Cirrhosis Cirrhosis is a chronic liver disease with progressive loss of the normal architecture of the liver tissue due to increasing fibrosis and scar tissue generation. The etiology of the disease is viral hepatitis worldwide; however, excessive alcohol intake is the most common cause in Western countries.

Cirrhotic cardiomyopathy A specific type of cardiac dysfunction seen in patients with cirrhosis. Cirrhotic cardiomyopathy encompasses an impaired systolic function in response to circulatory stress and/or altered diastolic relaxation of the heart together with electrophysiological changes in the absence of other known cardiac disease.

Hepatic encephalopathy Hepatic encephalopathy constitutes an altered level of consciousness or coma occurring as a result of liver failure. When the liver function deteriorates, toxic substances including ammonium are not cleared from the circulation resulting in increased concentrations passing the blood-brain barrier.

HRS The hepatorenal syndrome is a specific type of renal failure occurring in patients with advanced cirrhosis. Infections and cardiac dysfunction seem to precipitate the development of this severe complication, which, if untreated, has a high mortality.

Hyperdynamic circulation The hyperdynamic circulation is defined by an increased heart rate, cardiac output, and plasma volume together with a reduced arterial blood pressure and systemic vascular resistance. These changes occur in patients with advanced cirrhosis due to portal hypertension and vasodilatation.

Portal hypertension The portal vein delivers blood from the gastrointestinal tract back to the central circulation through the liver. The pressure in the portal vein increases as the amount of fibrosis and scar tissue in the liver increases, thereby increasing the resistance in the liver. Therefore, patients with advanced cirrhosis develop portal hypertension. The pressure in the portal vein can be assessed by liver vein catheterization, and a hepatic venous pressure gradient >5 mmHg designates portal hypertension.

RAAS The renin-angiotensin-aldosterone system is a hormonal system, which operates in order to secure sufficient blood flow to all vital organs of the body. The system is activated by sensors in the central blood vessels in case of low central blood pressure, and if activated the system induces vasoconstriction and sodium-water retention in the kidneys.

SBP Spontaneous bacterial peritonitis designates the development of infection in the abdominal cavity despite the absence of any obvious source of infection. Cirrhotic patients with ascites or variceal bleeding are prone to develop this type of infection.

Introduction

Liver cirrhosis is a chronic disease in which the normal architecture of the liver is lost because of a progressive fibrosis and formation of regeneration nodules. The most common underlying etiology worldwide is viral hepatitis, but in the Western countries excessive alcohol intake is the major cause of the disease. When the disease advances, the patients develop portal hypertension and splanchnic and systemic vasodilatation leading to a hyperdynamic circulation. These generalized circulatory changes may cause the development of severe complications such as ascites, esophageal varices, renal impairment, and hepatic encephalopathy. Appearance of these complications is associated with a high morbidity and mortality (Sanyal et al. 2008). In the last decade there has been increasing attention to the impact of cirrhosis on the heart, since both structural and functional cardiac

changes have been reported and more importantly seem to be related to the development of renal failure and to a poor prognosis (Krag et al. 2010a). Cardiovascular biomarkers have been associated to both the hyperdynamic circulation and the cardiac dysfunction and prognosis in these patients (Wiese et al. 2013a). This book chapter seeks to describe the current knowledge within this field focusing on novel cardiovascular markers.

Hemodynamic Disturbances in Cirrhosis

In the early stages of cirrhosis, the general hemodynamic changes are minor, but as the liver disease progresses, both the systemic and the splanchnic vascular beds become increasingly affected with appearance of a general circulatory dysfunction. The underlying pathophysiology consists of increasing fibrosis of the liver, which increases the intrahepatic resistance and generates portal hypertension. Portal hypertension contributes to both increased portosystemic shunting and the development of a splanchnic arterial vasodilation through an increased vasodilator activity, which may be brought about by a compensatory overproduction of vasodilators in the splanchnic vasculature. According to "the arterial vasodilation hypothesis," splanchnic arteriolar vasodilation leads to a progressive reduction of the systemic vascular resistance and central arterial underfilling with central hypovolemia, mainly due to vasodilators that escape degradation in the diseased liver or bypass the liver through the portosystemic shunts. Overall, the plasma and blood volumes are expanded in cirrhosis, but the distribution is unequal with a markedly reduction of the central blood volume. The blood volume denotes the combined blood volume of the heart, lungs, and central arterial tree and is a result of pooling of blood in the splanchnic and peripheral vascular territories (Henriksen et al. 1989). Low central blood volume combined with arterial hypotension activates potent vasoactive systems through volume and baroreceptors and hence the development of a hyperdynamic circulation. These systems include the renin-angiotensin-aldosterone (RAAS), the sympatho-adrenergic, and the vasopressin system and facilitate sodium and water retention in the kidneys and thereby plasma volume expansion (Schrier et al. 1988). Furthermore, the vasoconstrictor systems mediate vasoconstriction in the kidney with an increased risk of development of renal failure – the hepatorenal syndrome (HRS) (Arroyo and Colmenero 2003) (Figs. 1 and 2).

The hyperdynamic circulation is characterized by an increased heart rate, cardiac output, and plasma volume together with a low systemic vascular resistance and arterial blood pressure. The typical clinical features of the hyperdynamic circulation are reddish skin, palmar erythema, and a raised and bounding pulse, caused by the increased cardiac output and the pronounced vasodilatation (Wiese et al. 2013b). The increased cardiac output is attributed to the increased heart rate, increased venous return, and myocardial contractility. Additionally, the expanded blood volume, systemic vasodilatation, the presence of arteriovenous communications, and increased sympathetic nervous activity may further raise the cardiac

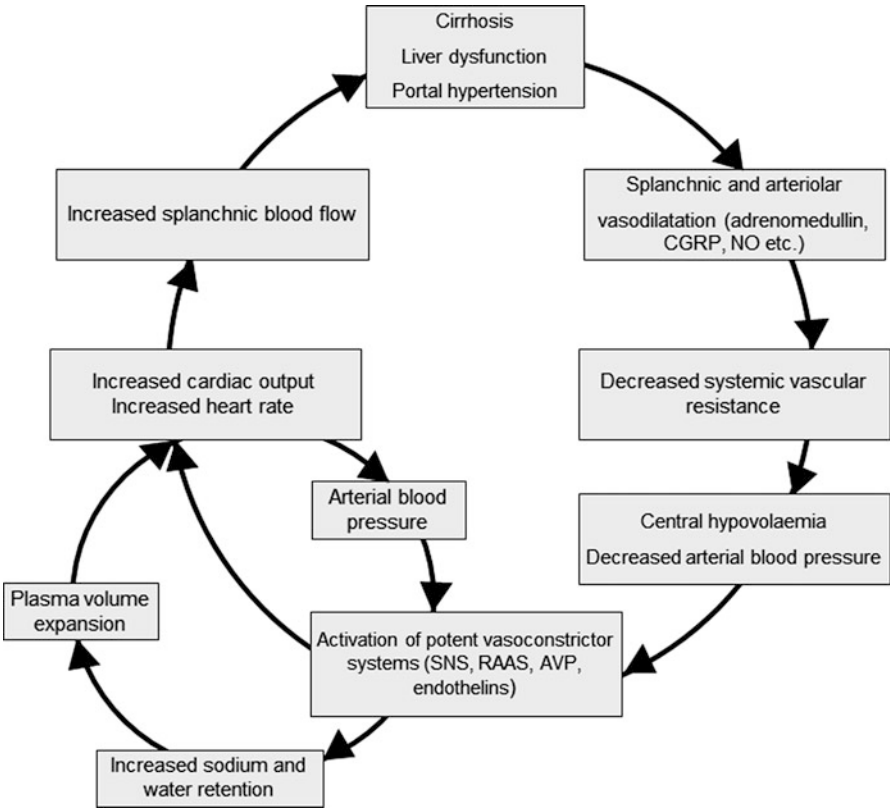

Fig. 1 The pathophysiology of the circulatory changes in cirrhosis. The splanchnic vasodilatation is induced by endogenous vasodilators escaping hepatic degradation, owing to portosystemic shunting and/or hepatocellular damage. Reduced systemic vascular resistance leads to a reduced effective arterial blood volume and activation of vasoconstrictor systems. The hemodynamic consequences are increases in heart rate, cardiac output, and plasma volume and decreased renal blood flow, low arterial blood pressure, and sodium and water retention. The development of the hyperdynamic circulation may increase portal inflow and further aggravate the portal hypertension in a vicious cycle. *CGRP* calcitonin gene-related peptide, *NO* nitric oxide, *SNS* sympathetic nervous system, *RAAS* renin-angiotensin-aldosterone system, *AVP* arginine vasopressin

output; most of these pathophysiological mechanisms are active in advanced cirrhosis (Iwakiri 2014). The increased cardiac output serves to compensate the effective hypovolemia; however, with disease progression the splanchnic vasodilatation becomes more pronounced, and this compensatory mechanism may be insufficient.

The hemodynamic changes in cirrhosis also include an impaired vascular reactivity, which conceivably contributes to the splanchnic vasodilatation and the abnormal distribution of the circulating blood volume. The impaired vascular reactivity is seen as an impaired response in blood pressure and blood flow to pharmacological stimulation with vasoconstrictors (angiotensin II or noradrenaline), changes in body

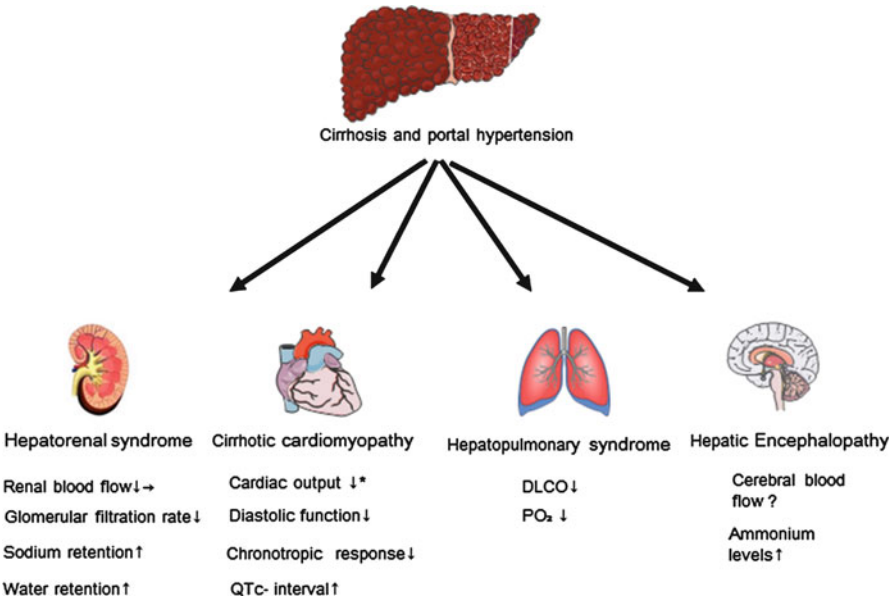

Fig. 2 Extrahepatic complications in cirrhosis. The systemic hemodynamic changes in advanced cirrhosis affect multiple organs including the kidney, heart, lungs, and brain leading to the development of severe complications such as the hepatorenal syndrome, cirrhotic cardiomyopathy, the hepatopulmonary syndrome, and hepatic encephalopathy. *DLCO* lung diffusion capacity, PO_2 arterial oxygen saturation. * The cardiac output in response to circulatory stress is decreased compared to healthy controls

position, plasma volume expansion, and exercise. The underlying mechanism of the vascular hyporesponsiveness is believed to be a combination of both a surplus of vasodilating substances such as nitric oxide and autonomic dysfunction (Trevisani et al. 1999). Several studies of the hemodynamic changes in cirrhosis have documented a high prevalence of autonomic dysfunction, related to liver dysfunction, portal hypertension, and survival (Ates et al. 2006).

Autonomic dysfunction encompasses chronotropic incompetence, increased sympathetic nervous activity, and decreased baroreflex sensitivity. Since these mechanisms greatly influence on the systemic hemodynamics, it has been speculated that autonomic dysfunction is also associated with the cardiac dysfunction in cirrhosis. Recent experimental findings support this theory by showing an association between reduced baroreceptor sensitivity and myocardial remodeling including left ventricle hypertrophy and a relation between autonomic dysfunction and impaired myocardial distribution of sympathetic nervous activity. Furthermore, chronotropic incompetence may play a role in the occurrence of hepatic nephropathy (Song et al. 2012; Moller et al. 2012).

A number of potent intrinsic vasodilators are implicated in the hemodynamic changes in cirrhosis. Especially nitric oxide, calcitonin gene-related peptide, and adrenomedullin seem to play an important part (Hori et al. 1997; Guevara et al. 1998). Other substances with vasodilating properties, which have been

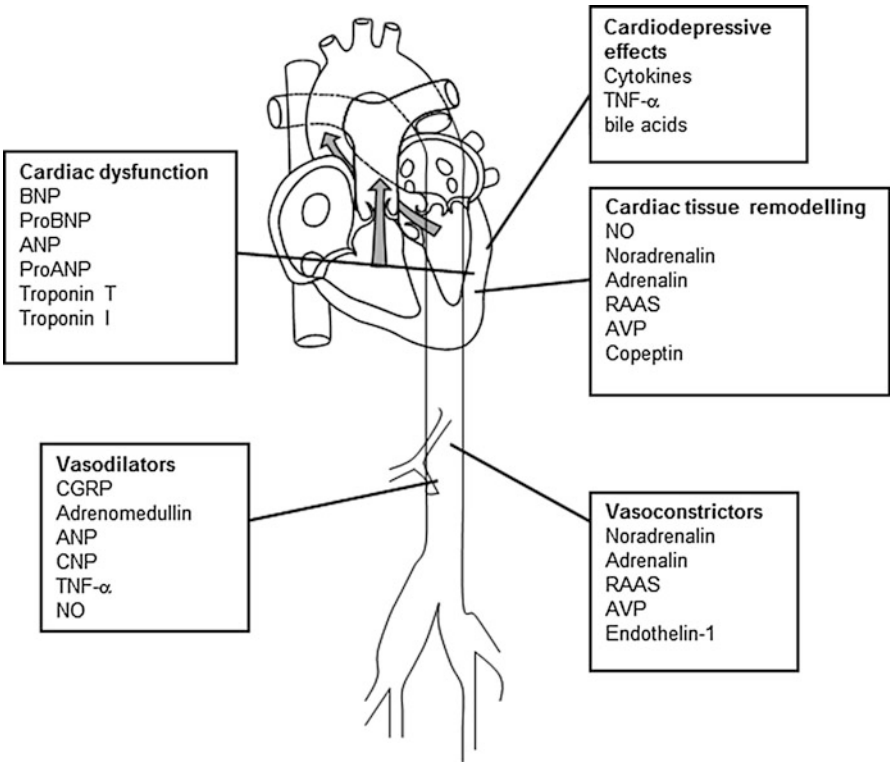

Fig. 3 Cardiovascular substances involved in the cardiac and circulatory dysfunction in cirrhosis. Vasoactive substances originating from the central nervous system, from the autonomic system, from local mediators, or within the smooth muscle cell/heart muscle cell contribute to the vascular hyporeactivity and to the development of cirrhotic cardiomyopathy. Vasodilators and vasoconstrictors may act variably at cardiac, arterial, and arteriolar levels. *BNP* brain natriuretic peptide, *proBNP* BNP prohormone, *ANP* atrial natriuretic peptide, *proANP* ANP prohormone, *CGRP* calcitonin gene-related peptide, *CNP* C-type natriuretic peptide, *TNF-α* tumor necrosis factor alpha, *NO* nitric oxide, *RAAS* renin-angiotensin-aldosterone system, *AVP* arginine vasopressin

shown to be increased, are natriuretic peptides, TNF-α, IL-6, and vascular endothelial growth factor (Iwakiri 2014) (Fig. 3).

Bacterial Translocation and Infections

Patients with cirrhosis are also more susceptible to bacterial infections. The primary cause is a reduced clearance of bacteria owing to an impaired function of macrophages and monocytes located in the liver, which are important cells of the cellular immune system, intra- and extrahepatic shunts, and deficiencies in the complement system (Fernández and Gustot 2012). Additionally, bacterial overgrowth of the gut and an increased intestinal permeability facilitate the translocation of bacteria from

the gastrointestinal tract to mesenterial lymph nodes. Especially the translocation of *Escherichia coli* from the gut plays a significant role for the development of spontaneous infections, in particular spontaneous bacterial peritonitis (Wiest et al. 2012). Bacterial translocation contributes to the hemodynamic disturbances by a marked increase in circulating levels of proinflammatory cytokines, following activation of monocytes and lymphocytes. The cytokines include TNF-α and IL-6 with subsequent activation of nitric oxide. This inflammatory response further augments the circulatory dysfunction and aggravates the vasodilatory state. In patients with cirrhosis, infection of the ascitic fluid – as seen in spontaneous bacterial peritonitis – is frequent, and it is an important risk factor for the development of circulatory and renal dysfunction (Ruiz-del-Arbol et al. 2003). Spontaneous bacterial peritonitis (SBP) is a major precipitating factor for the development of HRS as one-third of the patients with SBP develop HRS (Follo et al. 1994).

Cardiac Dysfunction in Cirrhosis

For years it was anticipated that impaired cardiac function in patients with cirrhosis was caused by the abuse of alcohol. However, within the recent years an increasing amount of evidence of a specific heart disease associated with cirrhosis has led to the definition of the entity *cirrhotic cardiomyopathy* (Liu et al. 2006). This condition has been reported in 40–50 % of cirrhotic patients, is independent of etiology with cardiac changes reported even in children with biliary atresia, and includes both functional and structural changes in the heart (Jones et al. 2010; Wiese et al. 2013a). According to the 2005 World Congress of Gastroenterology working definition, cirrhotic cardiomyopathy encompasses systolic dysfunction, diastolic dysfunction, and electromechanical abnormalities with prolonged QT interval in the absence of other known causes of cardiac disease (Moller et al. 2010) (Table 1). In cirrhotic patients, the cardiac dysfunction is often subclinical owing to a reduced afterload because of the pronounced systemic vasodilation which protects the cardiac function, whereas when the patient is facing situations with circulatory stress such as major surgery, variceal bleeding, or infections, the clinical manifestations may emerge with the risk of overt heart failure. It still remains unresolved whether cirrhotic cardiomyopathy is related to the severity of the liver disease, but there is some evidence hereof, since the most pronounced cardiac dysfunction is seen in decompensated cirrhosis (Ruíz-del-Árbol et al. 2013).

Structural Changes

The structural cardiac changes in cirrhosis seem to primarily affect the left side of the heart, and the two most pronounced features, which are also included in the definition of cirrhotic cardiomyopathy, are an increased myocardial mass and an enlarged left atrium. Hypertrophy of the left ventricle is documented in several studies and is

Table 1 The 2005 World Congress of Gastroenterology diagnostic and supportive criteria for cirrhotic cardiomyopathy

A working definition of cirrhotic cardiomyopathy
A cardiac dysfunction in patients with cirrhosis characterized by impaired contractile responsiveness to stress and/or altered diastolic relaxation with electrophysiological abnormalities in the absence of other known cardiac diseases
Diagnostic criteria
Systolic dysfunction
Blunted increase in CO with exercise, volume challenge, or pharmacological stimuli
Resting EF <55 %
Diastolic dysfunction
E/A ratio <1
Prolonged deceleration time (>200 ms)
Prolonged isovolumetric relaxation time (>80 ms)
Supportive criteria
Electrophysiological abnormalities
Abnormal chronotropic response
Electromechanical uncoupling
Prolonged QT interval
Enlarged left atrium
Increased myocardial mass
Increased BNP and proBNP
Increased troponin I

CO cardiac output, *EF* left ventricular ejection fraction, *E/A ratio* ratio of early to late (atrial) phases of ventricular filling, *BNP* brain natriuretic peptide

unrelated to the etiology of cirrhosis (Pozzi et al. 1997). A recent postmortem analysis of a large population of cirrhotic patients reported a high rate of cardiac abnormalities such as left ventricle hypertrophy and cardiomegaly in approximately one-third of the patients (Ortiz-Olvera et al. 2011). The underlying mechanisms appear to be a combination of the hyperdynamic circulation, activation of RAAS, and increased circulating levels of endotoxins, cytokines, and bile acids due to the impaired liver function, all facilitating myocardial remodeling. Additionally, the increased aldosterone release due to RAAS activation enhances myocardial fibrosis (Desai et al. 2010). The increase in the wall thickness together with fibrosis and subendocardial edema generates an increased stiffness and abnormal relaxation of the left ventricle, thereby possibly contributing to the diastolic dysfunction reported in cirrhotic patients (Liu et al. 2006). The enlarged left atrium is well documented and seems to be related to progressively advanced liver disease (Pozzi et al. 1997; Møller et al. 1995).

Systolic Dysfunction

Systolic dysfunction is defined as an inability of the heart to produce an adequate cardiac output and arterial pressure in order to meet the demands of the body. In

cirrhosis, the cardiac dysfunction has been expressed as a hyperdynamic unloaded failure of the heart. During rest the systolic function of the left ventricle measured as left ventricle ejection fraction (LVEF) is normal in the majority of cirrhotic patients (Wong 2009). The lack of systolic dysfunction during rest is most likely explained by the reduced afterload due to the low systemic vascular resistance, which reduces the workload and thereby protects the function of the left ventricle as mentioned previously. However, contemporary echocardiographic techniques have increased the possibility to detect very early stages of systolic dysfunction (meaning before LVEF is affected) by evaluating the deformation of the heart during the cardiac cycle, and therefore it is now possible to detect early signs of systolic dysfunction at rest (Wiese et al. 2013b). The cardiac dysfunction in cirrhosis primarily encompasses an inability to raise cardiac output sufficiently in response to stress testing. Exercise, volume challenge, or vasoconstrictive drugs are various methods of mimicking a situation with increased circulatory stress. Accordingly, when cirrhotic patients are exposed to exercise, the filling pressure of the left ventricle increases and is followed by a less than normal increase in LVEF and heart rate, respectively. Administration of terlipressin results in an increase of the afterload and in the end-diastolic volume of the left ventricle, but a decrease in cardiac output (Krag et al. 2010b). Furthermore, infusion of angiotensin II elicits an abnormal response including a 30 % increase in afterload with doubling of the pulmonary capillary wedged pressure without changes of the cardiac output (Limas et al. 1977). The systolic dysfunction in cirrhosis may have an impact on the development of complications, such as sodium and water retention, ascites formation, development of HRS, and prognosis. Especially, the relation to HRS has been investigated thoroughly, and a low cardiac output seems to be associated with an increased risk of developing HRS (Krag et al. 2010b, Gut 2010).

Diastolic Dysfunction

Diastolic dysfunction of the heart constitutes an abnormal relaxation and filling pattern of the left ventricle. It is the most pronounced feature of cirrhotic cardiomyopathy and is reported in about half of the patients with cirrhosis. Moreover, diastolic dysfunction appears to be most prominent in patients with advanced stages of cirrhosis (Pozzi et al. 1997). The clinical significance of diastolic dysfunction in cirrhotic patients has been questioned, as overt cardiac failure is not a prominent feature of cirrhosis. However, there are several reports of unexpected death from heart failure following surgical procedures with a rapid increase in cardiac preload such as liver transplantation, surgical portacaval shunts, and TIPS (Myers and Lee 2000; Ginès et al. 2002). In the presence of diastolic dysfunction, the heart becomes less compliant due to an increased stiffness of the myocardial wall; therefore, pulmonary edema and heart failure may arise. The pathological basis of the increased stiffness of the left ventricle seems to be cardiac hypertrophy, patchy fibrosis, and subendothelial edema (Lossnitzer et al. 2010). Additionally, diastolic dysfunction seems related to the development

of ascites, HRS, and impaired survival, although these relations need to be confirmed in larger prospective trials (Ruiz-del-Arbol et al. 2013). Diastolic dysfunction is easily visualized by echocardiography and is characterized by a decreased E/A ratio and delayed early diastolic transmitral filling with prolonged deceleration and isovolumetric relaxation times together with the corresponding characteristics on the tissue Doppler and speckle echocardiography (Kazankov et al. 2011; Sampaio et al. 2013).

QT Interval Prolongation

Conductance abnormalities are frequent observed in patients with cirrhosis (Bernardi et al. 1998). Especially, a prolonged QT interval is prominent and is significantly related to the severity of the liver disease, portal hypertension, autonomic dysfunction, and reduced survival (Henriksen et al. 2003). Recently, it has been documented that acute gastrointestinal bleeding further prolongs the QT interval and that a prolonged QT interval is an independent predictor of bleeding-induced mortality in cirrhosis (Trevisani et al. 2012). The prolonged QT interval in cirrhosis should be considered an element in the cirrhotic cardiomyopathy and may be of potential clinical relevance in the identification of patients at risk. The prolongation of the QT interval is partly reversible after liver transplantation and beta-blocker treatment (Mohamed et al. 1996).

Extrahepatic Complications in Advanced Cirrhosis

During the last decade it has become clear that chronic liver failure not only is limited to a decreased liver function but also involves impairment of most other organs in the body as part of a multi-organ syndrome particularly relating to hemodynamic and homeostatic disturbances (Møller et al. 2014). The most severe complications comprise formation of ascites, bleeding from esophageal varices, renal failure (HRS), and hepatic encephalopathy (Fig 2). The combination of portal hypertension and the hyperdynamic circulation plays an essential role in the underlying pathophysiology of these conditions (D'Amico et al. 1986). Increased portal pressure, alterations in the intestinal capillary pressure and permeability, and activation of RAAS, the sympatho-adrenergic system, and the vasopressin system are all involved in the accumulation of fluid within the abdominal cavity as ascites and in the impairment of the renal function with marked decreases in renal excretion of free water and sodium. When the patients develop ascites, they are categorized as having decompensated cirrhosis. As the liver disease deteriorates, the patients become more decompensated and the renal impairment progresses; hence, they develop dilutional hyponatremia and ultimately HRS. The prognosis of patients with ascites is poor with a 2-year mortality of more than 50 %, and if they develop HRS, the median survival is less than 1 month (Arroyo and Colmenero 2003). Bleeding from esophageal varices is caused secondary to the development of a massive collateral vein

formation and shunting of blood from the splanchnic to the systemic circulation. Furthermore, the shunting of blood to the systemic circulation in combination with impaired liver function leads to increased systemic levels of toxins and ammonia, which crosses the blood-brain barrier precipitating hepatic encephalopathy (Bismuth et al. 2011). Another complication, which is seen more rarely in cirrhotic patients, is pulmonary dysfunction or porto-pulmonary hypertension, a condition accompanied by a significant morbidity and mortality. The observed pulmonary changes are termed the hepatopulmonary syndrome (HPS) and consist of a reduced lung diffusing capacity, an abnormal ventilation/perfusion ratio, the presence of intrapulmonary shunts, decreased pulmonary vascular resistance, and low arterial oxygen saturation (Fallon and Abrams 2000).

Markers of Cardiovascular Dysfunction

Catecholamines

The sympathetic nervous system (SNS) is overactive in advanced stages of cirrhosis and seems to play a prominent role in the progressive circulatory dysfunction. Primarily, the increased levels of catecholamines affect the renal perfusion and sodium-water retention (Bendtsen et al. 1991a) since both noradrenaline and adrenaline are powerful vasoconstrictors (Table 2). The renal hypoperfusion and the increased sodium-water retention seem to precede the development of ascites and, in later stages, the development of HRS (Arroyo and Colmenero 2003). Moreover, the persistent elevated catecholamine levels appear to be involved in the pathogenesis of cardiac dysfunction most likely owing to the constant catecholamine stimulation that downregulates cardiac beta-adrenergic receptors and thereby impairs cardiac β-adrenoceptor signaling. In addition to downregulation, other mechanisms include desensitization and sequestration of β-adrenoceptors at the cell surface of the cardiomyocyte (Gerbes et al. 1986; Lee et al. 1990). The chronotropic incompetence (a defective capacity to increase heart rate during strain) seen in advanced cirrhosis seems to arise due to the abnormal β-adrenoceptor signaling. This condition has been related to disease severity, degree of portal hypertension, and poor prognosis (Ates et al. 2006). Furthermore, elevated plasma noradrenaline is directly associated to the QT interval prolongation (Bernardi et al. 1998).

The increased plasma levels of adrenaline and noradrenaline occur on the basis of both an increased release from the adrenal gland mediated by baroreceptors and an impaired hepatic extraction in the cirrhotic liver. In a healthy liver the extraction ratio of adrenaline and noradrenaline is very high and ranges 63–67 % (Henriksen 1991).

The Renin-Angiotensin-Aldosterone System

The pronounced activation of RAAS in advanced cirrhosis is believed to be a counter-regulatory mechanism due to the decreased arterial blood pressure and

Table 2 Vasodilatory and vasoconstricting forces involved in the disturbed hemodynamics in cirrhosis

Vasodilator factors
Adenosine
Adrenomedullin (ADM and proADM)
Atrial natriuretic peptide (ANP and proANP)
Bradykinin
Brain natriuretic peptide (BNP and proBNP)
Calcitonin gene-related peptide (CGRP)
C-type natriuretic peptide (CNP)
Carbon monoxide
Endocannabinoids
Endothelins
Endotoxins
Histamine
Interleukins
Nitric oxide (NO)
Prostacyclin
Substance P
Tumor necrosis factor-α (TNF-α)
Vascular endothelial growth factor (VEGF)
Vasoconstrictor factors
Angiotensin II
Endothelins
Adrenaline
Noradrenaline
Neuropeptide Y
Renin-angiotensin-aldosterone system (RAAS)
Sympathetic nervous system (SNS)
Vasopressin (AVP and copeptin)

low effective central blood volume registered by the baro- and volume receptor (Bernardi et al. 1990). Furthermore, the degradation of renin, angiotensin II, aldosterone, and vasopressin is impaired in the cirrhotic liver leading to the increased plasma levels of these highly vasoactive substances (Henriksen 1991). The hemodynamic consequences of RAAS activation involve both increases in cardiac output, heart rate, and plasma volume and decreased renal blood flow and sodium-water retention. Especially, RAAS appears to be involved in the development of renal failure (HRS), and cirrhotic patients with HRS have the highest levels of plasma renin. Moreover, elevated plasma renin activity independently predicts the development of HRS (Arroyo and Colmenero 2003). Activation of RAAS is associated with cardiac hypertrophy and heart failure (Danser et al. 1997). In patients with advanced cirrhosis, the excessive RAAS activity also seems related to diastolic dysfunction and may possibly induce myocardial fibrosis (De et al. 2003, Raizada et al. 2007). Therefore, the potential role of RAAS in cardiac dysfunction in cirrhosis needs to be further elucidated.

Vasopressin

In advanced cirrhosis there is an increased release of arginine vasopressin (AVP) – also known as the antidiuretic hormone. The release of AVP is caused by stimulation from endotoxins and proinflammatory cytokines following bacterial translocation in the gut. However, AVP is rapidly degraded in the circulation, which limits its potential as a prognostic marker (Møller et al. 2001). Instead, copeptin has emerged as a prognostic marker of an increased AVP activity, because copeptin is derived from the prohormone of AVP, is secreted equimolarly with AVP, and undergoes less degradation in the circulation. Copeptin concentrations increase with disease severity in cirrhosis and even further if the patients develop sepsis (Moreno et al. 2013). AVP has been shown to worsen the cardiac dysfunction in patients with congestive heart failure by increasing cardiac preload and afterload, as well as causing myocardial ischemia and remodeling (Schrier 2006), and copeptin has been associated with a poor outcome in these patients. In cirrhotic patients with a low cardiac output, copeptin levels are also increased; hence, AVP may also be involved in the pathogenesis of cirrhotic cardiomyopathy (Wiese et al. 2013a). Moreover, there seem to be several associations between copeptin and both splanchnic and systemic hemodynamics, hence indicating that AVP is involved in the circulatory dysfunction in cirrhosis (Kimer et al. 2014). Copeptin also seems related to the presence of ascites; thus, AVP may possibly be involved in the complex pathophysiology leading to ascites formation (Sola et al. 2013). Finally, copeptin also seems to be directly related to risk of death or liver transplantation (Moreno et al. 2013). In conclusion, copeptin seem to have potential as an important marker of cardiac dysfunction and poor outcome in cirrhotic patient, but more research on these relations are warranted.

Endothelins

The pronounced arterial vasodilatation in advanced cirrhosis leads to activation of counter-regulatory mechanisms including activation of RAAS and the sympathetic nervous system and increased release of copeptin as mentioned above. Additionally, it may include the release of endothelins. The combined actions of all these systems are anticipated to be major players in keeping the arterial blood pressure almost within normal range (Møller et al. 2001). Endothelins are peptides with very potent vasoactive properties located in the vascular endothelium and in various organs including the liver. In cirrhosis, especially endothelin-1 has attracted interest with the finding of elevated plasma levels with the highest levels reported in decompensated patients and associations to disease severity and liver dysfunction (Moore et al. 1992). Endothelin-1 is a known potent vasoconstrictor and has also been related to the arterial blood pressure in cirrhosis suggesting involvement in the blood pressure regulation (Helmy et al. 2001). However, as endothelin-1 has also been shown to induce vasodilatation in patients with advanced cirrhosis (Vaughan et al. 2003), the role of endothelin-1 in the pathophysiology of the systemic

vasodilatation of cirrhosis remains somewhat complex. Endothelins may also contribute to the development of portal hypertension, as activated hepatic stellate cells synthesize endothelin-1 and may contribute to the increased resistance to portal flow in the cirrhotic liver (Pinzani et al. 1996). Furthermore, a recent study has hypothesized that RAAS and endothelin-1 may be involved in the pathogenesis of liver fibrosis (He et al. 2013). In conclusion, the endothelin system seems involved in the pathogenesis of cirrhosis in several ways, which needs to be further investigated.

BNP and proBNP

Several studies have investigated the role of brain natriuretic peptide (BNP) in cirrhosis, and circulating levels of BNP are elevated in both patients with compensated and decompensated cirrhosis (Henriksen et al. 2003). The release of BNP is stimulated by myocardial strain of the left ventricle, and increased levels of BNP are associated with a poor survival in patients with cardiomyopathy, irrespective of etiology (McDonagh et al. 1998). In cirrhosis, BNP seems related to disease severity, cardiac dysfunction, and survival (Henriksen et al. 2003). Furthermore, BNP levels measured intraoperatively at liver transplantation are an independent predictor of 1-year all-cause mortality with a high negative predictive value, suggesting that it could be used for identification of patients with low risk of posttransplant mortality (Kim et al. 2011). The prohormone of BNP (proBNP) is also increased in cirrhotic patients and is associated with disease severity and poor survival (Henriksen et al. 2003; Wiese et al. 2013b) (Fig. 4). Elevated proBNP levels have been associated with cardiac dysfunction in cirrhosis, and a cutoff value of >290 ng/ml is highly predictive of diastolic dysfunction (Raedle-Hurst et al. 2008). Moreover, proBNP is associated with the presence of ascites and portal hypertension, and both BNP and proBNP are associated with the QT prolongation seen in cirrhosis (Henriksen et al. 2007). Patients with cirrhosis and porto-pulmonary hypertension also display significantly higher levels of proBNP; hence, proBNP might have potential as a noninvasive diagnostic test of this rare complication (Bernal et al. 2009). Another possible role of BNP could be in the initial workup of patients presenting with ascites, where it seems to be a potential differential diagnostic test, as a cutoff of >364 pg/mL has a high positive likelihood ratio of heart failure-related ascites (Farias et al. 2014).

ANP and proANP

Atrial natriuretic peptide (ANP) is secreted from the cardiac atria in response to myocardial injury and hypertrophy, and increased plasma ANP is associated with a poor survival (McDonagh et al. 1998). In cirrhosis circulating ANP levels are increased in decompensated patients, and ANP is related to the severity of liver function impairment (Cillo et al. 2001). ANP reduces renal plasma flow and the glomerular filtration rate in

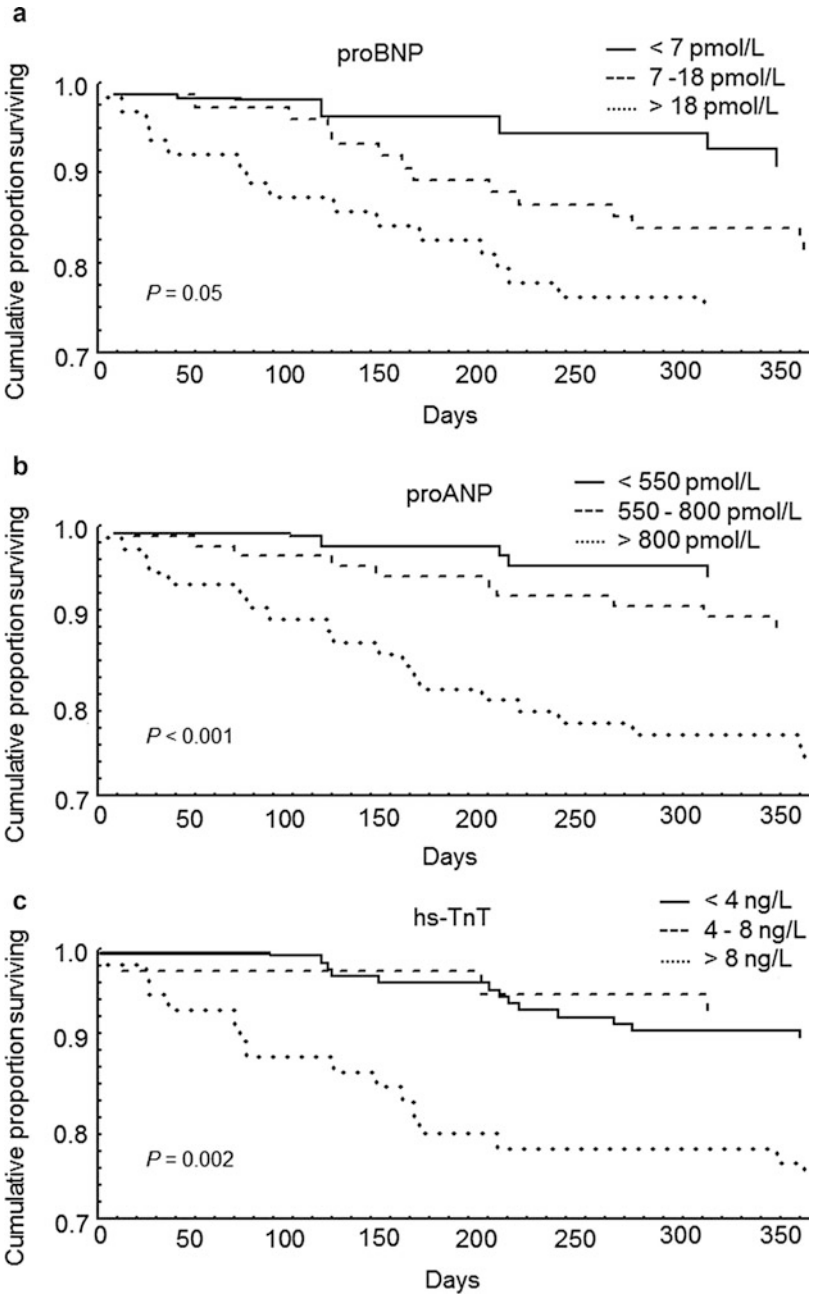

Fig. 4 Kaplan-Meier plots of 1-year survival in relation to cardiac markers. The panels show 1-year survival in relation to circulating levels of brain natriuretic peptide prohormone (proBNP) (panel **A**), atrial natriuretic peptide prohormone (proANP) (panel **B**), and high-sensitivity troponin T (hs-TnT) (panel **C**) with cirrhotic patients divided into three strata of approximately equal size

cirrhotic rats. Therefore, ANP seems to play an important role in the development of renal failure in cirrhosis (Angeli et al. 1994). Recently, the prohormone of ANP (proANP) has been suggested as a better prognostic marker than ANP due to the following: proANP is co-secreted with ANP and undergoes less enzymatic degradation and less receptor binding than ANP, hence giving higher and more stable circulating plasma levels. Increased levels of proANP have been shown to be associated with disease progression and increased mortality in patients with congestive heart failure (Masson et al. 2010, Miller et al. 2012). In cirrhosis circulating proANP levels are also increased and seem related to disease severity (Wiese et al. 2013a) (Fig. 4). This is in keeping with earlier observations that cardiac dysfunction is more frequent and pronounced in patients with more advanced stages of the disease. In a recent study proANP seems related to portal hypertension and systemic hemodynamics (Kimer et al. 2014). This could indicate that cirrhosis is inducing cardiac strain – thus making it an important marker of cardiac dysfunction. However, in another study proANP was not related to cardiac output, but was associated with a poor long-term survival in patients with cirrhosis (Wiese et al. 2013b). Conclusively, the role of proANP in cirrhosis needs to be further elucidated.

CNP

The last group of the natriuretic peptides is C-type natriuretic peptide (CNP) and its prohormone (proCNP). CNP is widely expressed in the vasculature, especially in the endothelium, where it induces vasorelaxation and vascular remodeling (Møller et al. 2001). Circulating inflammatory cytokines and endotoxin stimulate the release of CNP (Suga et al. 1993). Hence, bacterial translocation seems to play a prominent role in the increased levels of CNP in cirrhosis. In animal models of cirrhosis, the upregulation of CNP has been related to vasodilatation, thereby contributing to the hyperdynamic circulation (Komeichi et al. 1995). Few studies have investigated CNP and proCNP in patients with cirrhosis; CNP appears to be involved in the sodium-water disturbances (Gülberg et al. 2000), whereas proCNP seems associated to complications, such as ascites, hepatic encephalopathy, and esophageal varices. In addition proCNP may also serve as an independent predictor of mortality or liver transplantation (Koch et al. 2012).

Troponin

Cardiac troponin I and troponin T (TnT) are another type of cardiovascular markers, which may also have prognostic potential in cirrhosis. They are both filament-

Fig. 4 (continued) using round cutoff values of the variable in question, and the probability curves are compared by the log-rank test (Data from ref. (Wiese et al. 2013b) with permission from publisher John Wiley and Sons Ltd)

associated proteins in the cardiac muscle, and the plasma concentrations increase when the myocardium is injured. Moreover, nonischemic conditions such as heart failure, left ventricular hypertrophy, chronic kidney disease, and diabetes may also cause increased plasma levels of TnT (Wallace et al. 2006). The new high-sensitivity TnT (hs-TnT) assay has been proposed as a better predictor than the established troponin assays with respect to the development of heart failure and survival (de Lemos et al. 2010). In cirrhosis, circulating levels of cardiac troponin I are reported increased in some patients showing signs of subclinical myocardial injury with a lower stroke volume and left ventricular mass index (Pateron et al. 1999). Moreover, elevated troponin I >0.1 ng/mL seems associated with the development of hepatic encephalopathy and a poor prognosis in patients with acute liver failure (Parekh et al. 2007). Cardiac troponin T has primarily been investigated in relation to liver transplantation. Patients with an elevated TnT >0.11 ng/mL postoperatively had both a higher cardiovascular and overall mortality compared with patients without TnT elevations; hence, TnT may help to risk stratify patients in the early postoperative setting (Snipelisky et al. 2013). Recently, hs-TnT has also been investigated in cirrhotic patients with the finding of elevated circulating concentrations of hs-TnT in patients with advanced liver disease. Although, hs-TnT was not related to an impaired cardiac function, it remained a strong independent predictor of a poor long-term outcome (Wiese et al. 2013a) (Fig. 4). Consequently, larger prospective studies are warranted in order to further determine the potential of cardiac troponins T and I as noninvasive markers of cardiac dysfunction and as prognostic markers in cirrhosis.

Calcitonin Gene-Related Peptide

The circulatory dysfunction in cirrhosis is primarily elicited by arterial vasodilatation, and several potent vasodilators appear to be important in the process. Calcitonin gene-related peptide (CGRP) is one of the most potent vasodilators known on a molar basis, and plasma CGRP is increased in patients with cirrhosis with the highest levels reported in patients with advance disease (Table 2). Moreover, circulating CGRP is related to liver dysfunction and systemic hemodynamics including cardiac output, systemic vascular resistance, and arterial compliance (Bendtsen et al. 1991). In experimental studies, antagonists of CGRP partly reverse the hyperdynamic circulation and arterial vasodilatation in cirrhotic rats (Hori et al. 1997).

Adrenomedullin

Adrenomedullin is a peptide similar to CGRP also with potent vasodilating effects and is primarily increased in patients with decompensated cirrhosis. In these patients, adrenomedullin correlates with liver dysfunction and indicators of vasoconstrictor systems like SNS, RAAS, and endothelin (Guevara et al. 1998). Similarly, the prohormone of adrenomedullin (proADM) seems to increase with disease

severity in cirrhosis and is related to splanchnic and systemic hemodynamics (Kimer et al. 2014). This indicates that adrenomedullin is involved in the circulatory dysfunction in cirrhosis.

Nitric Oxide, Vascular Endothelial Growth Factor, and Cytokines

Other vasoactive substances do also seem to play a role in the imbalance between vasodilating and vasoconstricting forces leading to the circulatory derangement in advanced cirrhosis (Table 2). There is a growing body of evidence that systemic nitric oxide production is increased and precedes the development of the hyperdynamic circulation in cirrhosis, thereby playing a major role in the arteriolar and splanchnic vasodilation and vascular hyporeactivity. Blockade of nitric oxide formation significantly increases arterial blood pressure and decreases plasma volume and sodium retention in both animal model and patients with cirrhosis (Martin et al. 1998). Furthermore, nitric oxide together with cardiodepressant substances such as cytokines, endothelins, and bile acids seems to contribute to the cardiac dysfunction by activation of cellular signaling pathways in the cardiomyocyte leading to reduced cardiac contractility as well as the abnormal function of the cardiac myofilaments (Wiese et al. 2013b). Vascular endothelial growth factor (VEGF) also seems involved in the hyperdynamic circulation, as VEGF stimulates angiogenesis and the development of portosystemic collaterals. In experimental models the blockade of the VEGF receptor-2 has been shown to inhibit this process and to revert portal hypertension and improve circulation. It has also been speculated that VEGF may be implicated in the development of specific complications in cirrhosis including the hepatopulmonary syndrome (Zhang et al. 2009). In addition inflammatory activation has been shown to aggravate circulatory failure. When patients with cirrhosis and SBP develop HRS, it has been hypothesized that the aggravation of the circulatory dysfunction is promoted by cytokines. Increased circulating levels of interleukins and TNF-α may further stimulate the RAAS and the sympathetic nervous system and thereby increased the renal vasoconstriction, thus resulting in a decreased glomerular filtration. Especially, TNF-α and IL-6 are reported elevated in plasma and in ascitic fluid of patients with SBP and related to a marked increase in RAAS activity (Arroyo and Colmenero 2003). Moreover, extremely high plasma levels of TNF-α are documented in patients with SBP and HRS, and TNF-α is related to an impaired cardiac output, RAAS, and the sympathetic nervous system in these patients (Ruiz-del-Arbol et al. 2003). However, the exact mechanism of the deterioration of circulatory function in SBP still remains unknown.

Potential Applications to Prognosis, Other Diseases, or Conditions

Markers of cardiac dysfunction may also contain prognostic information in patients with decompensated cirrhosis, since the circulatory changes occurring in the advanced stage are in analogy with those reported in congestive heart failure. In

particular ProBNP, proANP, and troponin, which are widely applied in the management of patients with heart failure and ischemic heart disease, may have prognostic potential in patients with cirrhosis. These markers are all increased in patients with decompensated cirrhosis and have all been associated with an increased mortality. Especially hs-TnT seems to be a strong independent predictor of a poor prognosis and may be a supplement to the prognostic assessment as obtained by the model for end-stage liver disease (MELD) score. MELD is a well-established scoring system among others for determining the prognosis in cirrhosis. In a recent study, the risk of dying within 1 year predicted by the MELD score was increased by a factor 1.6 if the plasma levels of hs-TnT was 4–8 ng/L and by a factor of 2.7 if they were increased to more than 8 ng/L (Wiese et al. 2013a). Furthermore, cirrhotic patients with a low cardiac output have increased levels of proBNP and copeptin, both of which are associated with a poor outcome in patients with congestive heart failure. These cardiac markers may therefore be used as a noninvasive method in order to evaluate cardiac dysfunction in cirrhosis. In patients undergoing liver transplantation, troponin, BNP, and copeptin have shown to be independent predictors of cardiovascular complications and mortality postoperatively, wherefore these markers may play an important role in the risk stratification (Snipelisky et al. 2013). The circulatory pathology of advanced cirrhosis and congestive heart failure share several features including the activation of the neurohumoral systems including AVP, RAAS, and the sympathetic nervous system. Furthermore, renal sodium and water retention lead to the development of complications such as edema, ascites, and pulmonary edema. The underlying mechanism in cirrhosis is a relative arterial underfilling due to the pronounced systemic arterial vasodilatation, whereas in congestive heart failure, there is an absolute arterial underfilling due to the decreased cardiac output. The activation of these counter-regulatory mechanisms is initiated in order to sustain arterial perfusion, but may also contribute to increased morbidity. Increased concentrations of aldosterone, noradrenaline, and AVP together with increased plasma renin activity have all been associated with poor outcome in patients with cardiac failure (Schrier 2006). Similarly, patients with non-cirrhotic cardiomyopathy are also reported to have elevated cytokine levels, but the role of cytokines in cardiac disease is beyond the scope of this review.

Conclusion

Liver cirrhosis is a chronic disease with pronounced hemodynamic changes affecting both the systemic and the splanchnic circulation. These circulatory changes lead to the development of extrahepatic complications involving numerous organ systems as a multi-organ failure syndrome. Especially, the development of ascites and renal failure (HRS) is related with a poor prognosis. The function of the heart is disturbed in cirrhosis, and cardiac performance is clearly impaired and seems to contribute to other complications such as renal failure. The arterial vasodilatation and the effective hypovolemia activate potent counter-regulatory vasoconstrictor systems such as RAAS, the sympathetic nervous system, and AVP. Similarly, the portal, splanchnic,

and systemic changes together with the increase inflammation and bacterial translocation facilitate the release of several vasoactive substances. A vast number of vasoactive substances reflect the hemodynamic changes in cirrhosis, and the recent years have considerably improved our understanding of the pathophysiology of natriuretic peptides, endothelins, CGRP, adrenomedullin, and nitric oxide in the circulatory derangement in cirrhosis, focusing on the imbalance between vasodilating and vasoconstricting forces. In the liver endothelin-1 synthesis seems both to be involved in the process of fibrosis and to contribute in the development of portal hypertension and in the systemic changes including renal vasoconstriction. Furthermore, natriuretic peptides and troponin are associated with the cardiac dysfunction in cirrhosis and may have potential as prognostic markers in patients undergoing liver transplantation. The cytokines released during inflammation may aggravate the circulatory dysfunction and possibly contribute to the development of renal failure, especially IL-6 and TNF-α which seem to be important in these processes. Although major questions remain unsolved, vasoactive substances play important roles in the clinical aggravation of circulatory and cardiac dysfunction and the development of renal complications. These aspects are important to take into account in the clinical managing of the cirrhotic patient and in the development of new drugs.

Summary Points

- Advanced cirrhosis is associated with the development of severe complications.
- Portal hypertension and pronounced arterial vasodilatation lead to generalized circulatory dysfunction.
- Cardiac dysfunction in cirrhosis may precede renal failure and affects the prognosis.
- There is an imbalance between vasoconstricting and vasodilating substances, and several vasoactive substances are involved.
- Potent vasoconstrictor systems are activated including the sympathetic nervous system, the renin-angiotensin-aldosterone system, and vasopressin system.
- Potent vasodilating substances are released including CGRP, adrenomedullin, and nitric oxide.
- Natriuretic peptides and troponin may have potential as prognostic markers including risk assessment in patients undergoing liver transplantation.

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Disease Focused Approach on Fibrosis Biomarkers in Cardiovascular Health

26

Michael A. Rosenberg

Contents

Abstract

Cardiac fibrosis is a marker of pathological remodeling and a risk factor for multiple conditions, in particular heart failure and cardiac arrhythmias. Although methods for detection of large-scale cardiac fibrosis are being developed using imaging modalities, these methods are limited in terms of both sensitivity for detection of low-level fibrosis and specificity for different pathways leading to fibrosis. A number of circulating biomarkers of mediators of the fibrosis process can be measured in the plasma, and the goal is that these markers might provide early detection of cardiac fibrosis or direct therapies toward specific pathological conditions. However, while several circulating fibrosis biomarkers hold promise

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_48

based on clinical investigations, more studies are needed before these factors are ready for application to clinical decision making.

Keywords

Fibrosis • Circulating biomarkers • Myocardial infarction • Atrial fibrillation • Cardiac arrhythmias • Cardiomyopathy • Prognosis • Sudden cardiac death

Abbreviations

AF	Atrial fibrillation
BMP-1	Bone morphogenetic protein-1
BNP (NT-proBNP)	Brain natriuretic peptide (N-terminal pro-brain natriuretic peptide)
CITP	C-terminal telopeptide, type I collagen
CTGF	Connective tissue growth factor
ECG	Electrocardiogram
ECM	Extracellular matrix
FN	Fibronectin
Gal-3	Galectin-3
HCM	Hypertrophic cardiomyopathy
HF	Heart failure
HFpEF	Heart failure with preserved ejection fraction (diastolic heart failure)
HFrEF	Heart failure with reduced ejection fraction (systolic heart failure)
ICD	Implantable cardioverter defibrillator
IL-(10,18,33)	Interleukin-(10,18,33)
LA	Left atrial
LGE	Late gadolinium enhancement
LV	Left ventricle
miRNA/mRNA	Micro-ribonucleic acid/messenger ribonucleic acid
MMP (1–13)	Matrix metalloproteinase (types 1–13)
MRI	Magnetic resonance imaging
NITP	N-terminal telopeptide, type I collagen
OPN	Osteopontin
PICP	Procollagen C-terminal peptide, type I collagen
PIIINP	Procollagen N-terminal peptide, type III collagen
PINP	Procollagen N-terminal peptide, type I collagen
RAAS	Renin-angiotensin-aldosterone system
SCD	Sudden cardiac death
SPARC	Osteonectin or “secreted protein acidic and rich in cysteine”
sST2	Soluble ST2
TGF-β	Transforming growth factor-beta
TIMP (1–4)	Tissue inhibitor of metalloproteinase (types 1–4)
TSP	Thrombospondin
VT/VF	Ventricular tachycardia/fibrillation

Key Facts About Circulating Fibrosis Biomarkers and Cardiovascular Disease

- Cardiac fibrosis plays an important part in several cardiac diseases.
- Through scientific discovery, investigators are now able to measure markers of the process of cardiac fibrosis in the blood.
- In heart failure, the pattern of fibrosis biomarkers released may be related to the function of the heart.
- Fibrosis biomarkers may be particularly useful for risk stratification in people with implantable cardioverter defibrillators (ICDs), who cannot undergo cardiac MRI.
- In atrial fibrillation, fibrosis biomarkers may be useful, although it is difficult to tell whether they are being released from the atria or ventricles in the heart.
- Circulating fibrosis biomarkers could potentially be useful to predict future cardiac disease and mortality, but more investigation, especially larger studies with greater numbers of biomarkers measured, is needed.

Definitions

Arrhythmia Abnormal electrical activity in the heart; generally categorized into problems with slow or impaired conduction (bradyarrhythmias) or problems with rapid or disorganized conduction (tachyarrhythmias). Tachyarrhythmias include "tachycardias," where electrical activity is organized, and "fibrillation," where electrical activity is disorganized.

Atrial fibrillation A cardiac condition in which the electrical activity in the atria is disorganized, causing the appearance of fibrillation on electrocardiogram (ECG).

Cardiac fibrosis Deposition of collagen in the heart, typically associated with pathological cardiac conditions.

Collagen A polypeptide formed from three alpha helices that makes up much of the connective tissue of the body. There have been 28 types described, although type 1 (fibrillar collagen) is most abundant.

Diastolic heart failure Also known as "heart failure with preserved ejection fraction," which refers to heart failure symptoms occurring when the ejection fraction of the heart is >50 %.

Late gadolinium enhancement A finding from contrast cardiac magnetic resonance imaging in which gadolinium is "held up" within the walls of the heart, typically used to identify areas of fibrosis.

Myocardial infarction A condition of acute myocardial cell death, typically resulting from obstruction to the coronary artery system, which if untreated can result in scar formation in the heart.

Pressure overload model Animal model in which the outflow from the left ventricle is partially constricted, leading to hypertrophy of the heart muscle; designed to mimic high blood pressure or aortic stenosis leading to diastolic heart failure.

Reactive fibrosis Fibrosis that occurs around living cells, typically due to activation of inflammatory pathways.

Replacement fibrosis Fibrosis that occurs in the setting of cell death, in which the "scar" formed replaces the previously living cells to maintain organ structure.

Sudden cardiac death By definition, refers to any sudden death in an individual previously observed to be healthy a brief period beforehand; generally, used to refer to death or near death due to lethal ventricular arrhythmias, ventricular fibrillation, or ventricular tachycardia.

Systolic heart failure Also called "heart failure with reduced ejection fraction"; refers to the cardiac condition in which the normal pump function (ejection fraction) becomes depressed.

Volume overload model Animal model in which excess blood is returned to the heart, causing it to dilate; designed to mimic systolic heart failure.

Introduction

Cardiac fibrosis plays an important part in cardiovascular health and disease. In studies of animal models of cardiac disease, studies of diseased human cardiac tissue, and imaging of human hearts in patients with cardiac disease, fibrosis has consistently been found to be present, and in many instances correlated with severity of the disease process. It has been associated with a number of cardiac risk factors, from advanced age to diabetes and obesity, and is one of the key mechanisms behind the development of many cardiac arrhythmias. Importantly, development of cardiac fibrosis plays a central role in the final stages of heart disease culminating in two causes of cardiac death: ventricular arrhythmias and heart failure (HF). For these reasons, there is active investigation into better methods to detect, quantify, and qualify cardiac fibrosis.

Until recently, the ability to examine cardiac fibrosis in humans was limited to studies in which cardiac tissue could be obtained, via cardiac biopsies or whole hearts obtained at the time of autopsy or cardiac transplant. The development of noninvasive methods to detect and quantify cardiac fibrosis, through advances in cardiac magnetic resonance imaging (MRI) technology as well as through measurement of circulating fibrosis factors in the blood, has opened a whole new realm of investigation into cardiac fibrosis in human cardiovascular disease. These studies

have raised important questions, both in terms of the biological significance of different patterns of cardiac fibrosis as well as the ability to identify clinical biomarkers of disease that might be used to risk stratify and target patients for treatment. Studies of "circulating fibrosis biomarkers" truly combine approaches from the "bench" with what can now be measured at the "bedside" and thus have great translational potential. However, these approaches are still in their infancy in terms of applicability, and to date, most studies of fibrosis biomarkers are as much about demonstrating feasibility and consistency as they are about identifying novel biology. As will be apparent throughout this review, there is a fair amount of conflicting data regarding which particular pattern of fibrosis biomarkers is seen in a given cardiac disease. Some of the conflict represents the natural limitations and variation when using small sample sizes, some of it may reflect the differing methodologies used to measure the biomarkers, and some may reflect the challenges with translational research, which requires investigators to compile knowledge and analytical approaches from the basic science world with those of clinical investigation.

Many of the bigger issues concerning measurement of biomarkers and disease in general will be covered elsewhere in this textbook. This review will specifically focus on circulating fibrosis biomarkers and their association with cardiovascular disease. For additional detail on circulating markers of collagen metabolism, see complimentary chapter in this textbook (Chalikias and Tziakas 2016). This chapter is organized to begin with a brief background on collagen metabolism, including the relevant metabolites that can be measured in the bloodstream. This background will also attempt to include other key molecular players in the cellular fibrotic process that have emerged from basic science studies and animal models; although many of these factors are not routinely or easily measured in the bloodstream, many are important regulators and worth mentioning. We will then delve into the clinical investigation of circulating fibrosis biomarkers for each of three key cardiovascular conditions in which fibrosis plays an integral part: heart failure, ventricular arrhythmias, and atrial fibrillation. For each condition, the reader will be provided some background on the significance of cardiac fibrosis for that particular disease, as well as the results from clinical studies of specific biomarkers.

Cardiac fibrosis in many ways signifies an endpoint in cardiovascular disease and is the result of a number of "upstream" processes. It would take an entire textbook to provide a comprehensive review of biomarkers of every pathological process that can lead to fibrosis. Among the topics that will not be covered in depth that are frequently studied closely with markers of fibrosis are biomarkers of inflammation (e.g., C-reactive protein), ischemia or infarction (e.g., troponin), and markers of myocardial wall stress and hemodynamics (e.g., natriuretic peptides, such as NT-proBNP). These topics are discussed elsewhere in this textbook, in addition to in a multitude of original studies and review articles. Another topic that will not be discussed is the emerging investigation of circulating miRNAs in the fibrotic process, although this is clearly an exciting area where active investigation is ongoing.

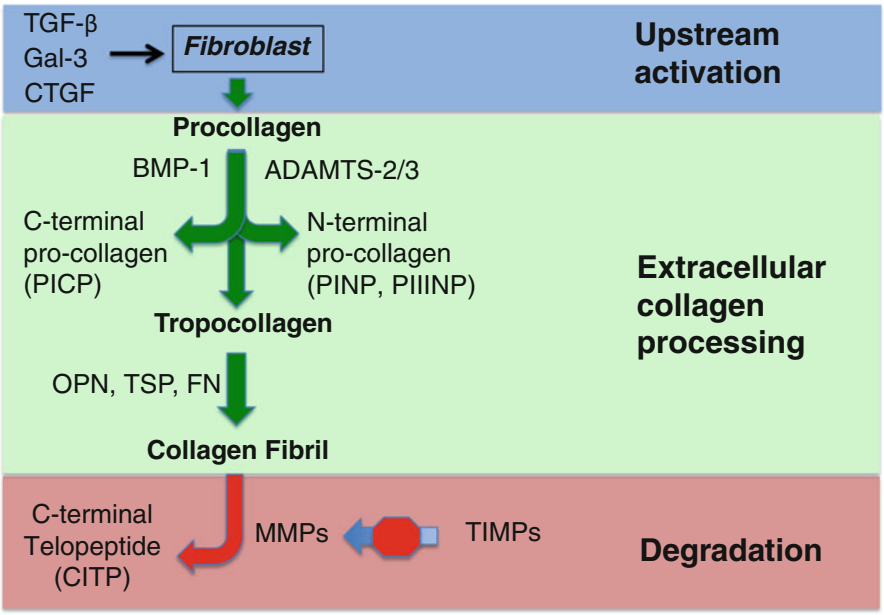
The Fibrotic Process

Fibrosis, or the deposition of collagen in the interstitial area surrounding cells, is among the most important processes in the human body, in that it not only provides a structural scaffold on which cells can grow and function but also because it provides a "repair" system for any physical damage that may take place over the life of an organism. Its significance is no less for the heart, where the connective tissue that makes up the cardiac skeleton transduces the force from contracting cardiomyocytes to allow the heart to pump blood to the vital organs. Through an active network of communication between cardiomyocytes, fibroblasts, and other non-cardiac cells within the *extracellular matrix* (*ECM*), the heart is able to monitor wall stress and oxygen balance and adjust the balance of pro- and anti-fibrotic factors to maintain homeostasis. More importantly, during periods of cardiac disease or damage, this system works to functionally repair the damage to allow continued organ function.

Like many biological processes, there are instances where the fibrotic response becomes excessive and pathological itself. Detection of pathological cardiac fibrosis has been the focus of many investigations; however, it is not always clear for many disease processes to what degree this fibrosis is part of a normal response to an ongoing insult (such as ischemia or wall stress) or the primary problem. In other words, fibrosis could actually be a marker of disease, rather than the problem itself. This concept is important to bear in mind, particularly when considering particular fibrotic pathways as targets of therapy.

Broadly, fibrosis is classified as either as *replacement fibrosis*, which occurs following cell death and loss of tissue, or *reactive fibrosis*, which occurs in the setting of inflammation triggered by a variety of stress conditions. In the heart, replacement fibrosis is what one would observe following a myocardial infarction, where due to disruption of blood flow in the coronary arteries, a large section of myocardium is destroyed. Following the initial ischemic insult, there is remodeling of the tissue in the area of the infarct, through which the dead cardiac cells are replaced by noncontractile, non-excitable cells and fibrous tissue. This replacement effectively "patches" the heart at the site of damage, replacing the previously functioning part of the heart with a nonfunctional scar, which can be detected with low-resolution cardiac imaging (i.e., transthoracic echocardiography) and often on the electrocardiogram (ECG). This process has been extensively studied, and much is known about the process of scar formation after a myocardial infarction.

Less is known, on the other hand, about the process of reactive fibrosis in the heart. At least in part due to heterogeneity in the conditions that lead to its development, reactive fibrosis seems to occur more indolently within the interstitial area of otherwise healthy cells, growing out from perivascular regions to infiltrate large areas of myocardium. It cannot be as easily detected with imaging or on ECG, but it can have profound effects on the normal function of the heart. At the level of basic investigation, discrimination of replacement from reactive fibrosis is not difficult based on the model of study; however, one should bear in mind that these distinctions are much more challenging in observational clinical investigations, which are where most of the circulating fibrosis biomarkers have been studied.



TGF-B: Transforming Growth Factor-Beta, **Gal-3:** Galectin-3, **CTGF:** Connective Tissue Growth Factor, **BMP-1:** Bone Morphogenic Protein-1, **ADAMTS-2/3:** A Disintegrin and Metalloproteinase with Thrombospondin Motifs-2/3, **OPN:** Osteopontin, **TSP:** Thrombospondin, **FN:** Fibronectin, **MMP:** Matrix Metalloproteinases, **TIMP:** Tissue inhibitor of metalloproteinase

Fig. 1 Fibrillar collagen (types I and III) metabolism. Simplified diagram of the metabolism of fibrillar collagen, highlighting factors relevant to cardiovascular disease and metabolites released into the circulation

The "fibrotic process" refers to the creation, deposition, and remodeling of collagen within the ECM. There are over 25 different types of collagen that have been described, although the two most important for cardiac fibrosis are the fibrillar collagens, types I and III. Others, such as type IV, which makes up much of the basement membrane in cells, are present in the heart, although less a focus of investigation as they are in other organs (i.e., kidney). Collagen I is the most abundant both within the heart, and the body at large, and is generally stiffer owing to thicker fibers with a greater degree of cross-linking. Collagen III is more reticular in nature, creating a network to provide structural integrity to the heart.

The metabolic process for both types I and III collagen is generally the same and begins in the *fibroblast cell* (and its derivative, *myofibroblast cell*), where the genes for collagens I and III (*COL1A1 and COL3A1*) are transcribed into mRNA and then translated into a pre-pro-peptide chain that undergoes a series of modifications, including hydroxylation of prolines and lysines in the Golgi apparatus (requires vitamin C as a cofactor), to create the triple helix *procollagen* molecule (Fig. 1). A number of factors play a role in the regulation of this process, with TGF-β playing

one of the central parts, although other members of the TGF-β family and downstream activators, such as connective tissue growth factor (CTGF), are also important.

Outside the cell, the soluble procollagen molecule is modified by *collagen peptidases*, which remove the "loose ends" on the C-terminal and N-terminal procollagen, rending it insoluble. The collagen peptidases include bone morphogenic protein-1 (BMP-1), which cleaves the procollagen C-terminal I peptide (**PICP**), and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-2/3, which cleaves the procollagen I (**PINP**) and procollagen III (**PIIINP**) N-terminal propeptides. PICP, PINP, and PIIINP are all released into the plasma, where they can be measured to quantify collagen formation. It should be noted that while PICP displays a 1:1 stoichiometry with type I collagen fibrils, PIIINP has been noted to occasionally be retained along with the type III collagen molecule, indicating that measurement of circulating PIIINP has the potential to underestimate the true amount of type III collagen fibrils in tissue (de Jong et al. 2011).

After cleavage of the C- and N-terminals, the collagen moiety is called *tropocollagen*. Tropocollagen is then linked by *lysyl oxidases* to form covalent bonds between the lysyl and hydroxylysyl groups and ultimately form the collagen fibril. This collagen fibril is modified by interaction with a number of other extracellular factors, such as osteopontin (**OPN**), osteonectin (also called "secreted protein acidic and rich in cysteine" or **SPARC**), fibronectin (**FN**), and thrombospondins (**TSP**s), which are secreted by macrophages. These factors play a vital role in coordinating the tissue fibrotic process and allowing communication between fibroblasts and immune/inflammatory cells. The significance of these factors in functional and pathological cardiac fibrosis has been demonstrated in a number of basic-level investigations. However, because few of these factors are easily measured in the peripheral circulation, their use as circulating fibrosis biomarkers has been limited.

Once deposited, fibrillar collagen does not remain permanently in place, but undergoes a coordinated remodeling process through interplay of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). There are four classes of MMPs: gelatinases (MMP-2 and MMP-9), collagenases (MMP-1, MMP-8, and MMP-13), stromelysin (MMP-3), and matrilysin (MMP-7). There are also four inhibitors of MMPs, the tissue inhibitors of metalloproteinases (TIMP-1, TIMP-2, TIMP-3, and TIMP-4). These factors play a complex role in fibrosis regulation (Spinale 2007). The collagenases (MMP-1, MMP-8, and MMP-13) break down fibrillar collagen and are inhibited through direct binding by the TIMPs in a 1:1 ratio. Any TIMP (1–4) can inhibit any MMP, although the specifics can differ based on the context. The gelatinases break down the products from the collagenases, but they also play a significant regulatory role. For example, MMP-9 activates a number of pro-fibrotic pathways, while MMP-2 is anti-fibrotic (de Jong et al. 2011; Spinale 2007). de Jong and colleagues point out that one of the challenges with interpretation of studies of MMPs is that most laboratory methods do not distinguish between free and active MMP and MMP that is inactive and bound to TIMPs (de Jong et al. 2011). This limitation may explain some of the mixed

and confusing results from studies of MMPs in cardiac disease and suggests the importance of using broad panel type of measurements (Zile et al. 2011), where levels of MMPs can be adjusted for level of TIMPs (MMP/TIMP ratio).

After digestion by MMPs, the fragments from the collagen molecule are broken off and released into the plasma, where they can be measured as a means to quantify collagen turnover. Among these fragments, N-terminal type I (**NITP**) and C-terminal type I (**CITP**) *telopeptides* are the most frequently measured.

To summarize, a number of collagen metabolites are released into the serum through the fibrotic process. PICP, PINP, and PIIINP are released in the formation of mature collagen and can thus be measured to quantify formation of new types I and III, respectively, in tissue. CITP and NITP are released in degradation of type I collagen and can be measured to quantify turnover of type I collagen in tissue. One can also measure the ratio of PICP to CITP as a method to quantify the state of collagen (type I) in the body, with a PICP/CITP >1 indicating net deposition of collagen I and a PICP/CITP <1 indicating a state of net collagen I remodeling. Relative levels of MMPs and TIMPs can also provide this type of information regarding the overall "fibrotic state" of a given organ, with an increased collagenase MMP (1, 8, or 13)/TIMP ratio generally indicating a net state of collagen breakdown.

In addition to the measurement of collagen metabolites, there are a number of additional upstream and downstream regulators of the process that have been measured as surrogates to the fibrotic process. Although some have emerged as being markers of collagen content and fibrosis, such as galectin-3, and soluble ST2 (discussed later), others, such as members of the renin-angiotensin-aldosterone system (RAAS), have wide-ranging effects on blood pressure, hemodynamics, and cardiac hypertrophy pathways, such that investigation of relationships with fibrosis is significantly more complicated. This group also includes inflammatory molecules, such as C-reactive protein and interleukins, which both directly and indirectly activate the process of cardiac fibrosis and can also be measured in the serum for correlation with cardiac fibrosis. However, their lack of specificity and indirect relationship with fibrosis makes them more complex to use as fibrosis biomarkers specifically. For simplicity and brevity, we will generally focus this review around the factors in the fibrosis pathway (i.e., collagen metabolism) that are correlated with cardiac disease states.

Cardiac Fibrosis and Cardiac Function

The primary function of the heart is to pump blood through the body and thereby provide oxygen and nutrients to vital organs, like the brain and kidneys. To accomplish their function, the cells of the heart have the unique qualities of both electrical excitability and contractibility, which enables them to communicate in order synchronize their contractions. In this manner, there is coupling of the electromechanical functions of the heart, and the heart is able to pump blood from the body to the lungs (right side) and from the lungs to the body (left side).

In health, the collagen present in the heart makes up the cardiac skeleton and valve structure and forms the basic structural apparatus of the functional organ. When pathological amounts of cardiac fibrosis develop, it disrupts the usual electromechanical function of the heart on a number of levels. In certain situations, such as following the death of a large number of ventricular cells due to myocardial infarction, the dense scar of replacement fibrosis replaces previously contractile tissue, leading to an increased burden of contractility on the remaining cardiac tissue, as well as increased wall stress due to an increase in chamber dimension/diameter, particularly in the setting of an aneurysm. In addition, the scar forms an electrically silent barrier to the normal propagation of the action potential through the heart, causing a number of arrhythmic conditions, in particular ventricular tachycardia.

When fibrosis occurs more indolently, in the reactive form that can result from any combination of low-level ischemia, wall stress, or inflammation, it can have similar consequences to those seen with replacement fibrosis (increased systolic dysfunction and risk of arrhythmias). In addition, since in interstitial fibrosis the fibrotic tissue is, by definition, interspersed between otherwise functional cardiomyocytes, there are other effects on cardiac function. For one, interstitial fibrosis inhibits the ability of the ventricular cells to relax during diastole, leading to increased diastolic filling pressures and eventually diastolic heart failure. During this process, the pressure transmitted to the atria also increases, frequently leading to increased atrial wall stress and increased risk of atrial fibrillation (Rosenberg et al. 2012b; Rosenberg and Manning 2012), which itself can increase atrial wall stress and the development of atrial fibrosis (Platonov et al. 2011).

Based on these mechanisms of cardiac pathology, investigations of circulating fibrosis biomarkers have generally focused on their use to detect cardiac fibrosis in situ leading to development of systolic and diastolic heart failure, ventricular arrhythmias and sudden cardiac death, and atrial fibrillation (Fig. 2). In the

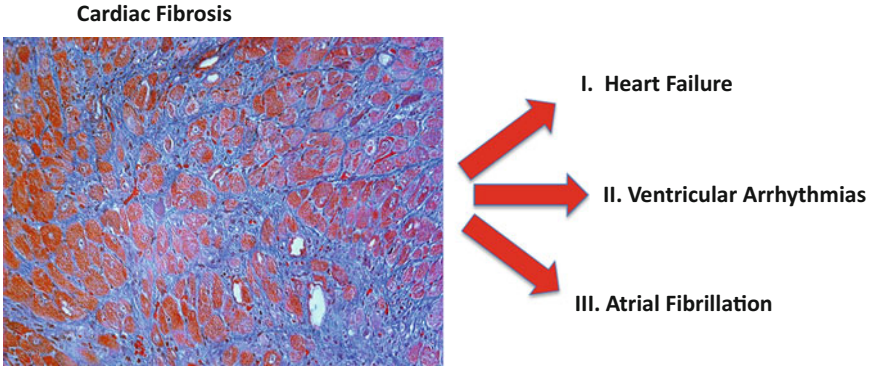


Fig. 2 Clinical outcomes associated with cardiac fibrosis. Masson's trichrome stain of mouse tissue demonstrating interstitial fibrosis after transverse aortic constriction model to induce heart failure. Three clinical associations (discussed in text) are heart failure, ventricular arrhythmias, and atrial fibrillation

remainder of this review, we will focus on each of these outcomes and the use of circulating fibrosis biomarkers in their analysis. For each condition, we will review the motivations behind study of cardiac fibrosis and fibrosis biomarkers and the results from clinical studies. We will also review some of the nuances and key limitations in these approaches, many of which result from the difficulties with peripheral measurements of circulating biomarkers described elsewhere in this textbook, but also from basic questions about how complex patterns of cardiac pathology play out in life.

Cardiac Fibrosis Biomarkers in Ventricular Conditions: Heart Failure

Heart failure (HF) is the pathological condition in which the heart is unable to adequately pump blood from the lungs to the body. Historically, the condition referred to a weakening of the heart's ability to contract ("systolic" HF); however, more recently, it has also been expanded to include states in which the heart contracts normally, but is unable to adequately relax ("diastolic" HF), leading to blood backed up into the lungs.

Systolic HF is generally categorized into two groups depending upon whether it is due to the loss of cardiac tissue due to a myocardial infarction (*ischemic cardiomyopathy*) or whether it is due to a generalized weakening of cardiomyocytes (*nonischemic*, *or dilated*, *cardiomyopathy*). From a fibrosis standpoint, these conditions are quite different as ischemic cardiomyopathies are generally associated with formation of a scar due to replacement fibrosis, while nonischemic cardiomyopathies have a variety of fibrosis patterns ranging from little or none up to fairly substantial amounts of fibrosis. There is still much to be learned in nonischemic cardiomyopathies about the causes for these patterns of fibrosis, but it is also an area in which the use of circulating fibrosis biomarkers might be useful.

Diastolic heart failure is not generally categorized in the same manner as systolic heart failure since many of the risk factors associated with it are less well defined. As a person ages, the ability of their heart to relax diminishes in general (Redfield et al. 2005). In addition, risk factors such as hypertension and obesity seem to play a role in the development of diastolic heart failure (Aurigemma 2006). Whether the mechanism for these risk factors directly involves development of cardiac fibrosis, or other mechanisms, remains to be determined. In certain situations, genetic mutations affecting cardiomyocytes can cause one or more walls of the ventricle to thicken, leading to a condition called hypertrophic cardiomyopathy, which is also associated with diastolic heart failure as well as sudden cardiac death (SCD). The latter association with SCD provides a key motivation for identifying biomarkers of risk, and fibrosis biomarkers are a group that investigators have examined.

To study cardiac fibrosis in heart failure, investigators have examined the heart tissue from both explanted human hearts, as well as from animal models designed to mimic these conditions. When the heart's pump function starts to fail, it undergoes a process called *eccentric remodeling*, in which the walls are stretched and thinned. In diastolic heart failure, the ventricle undergoes a process called *concentric remodeling*, in which

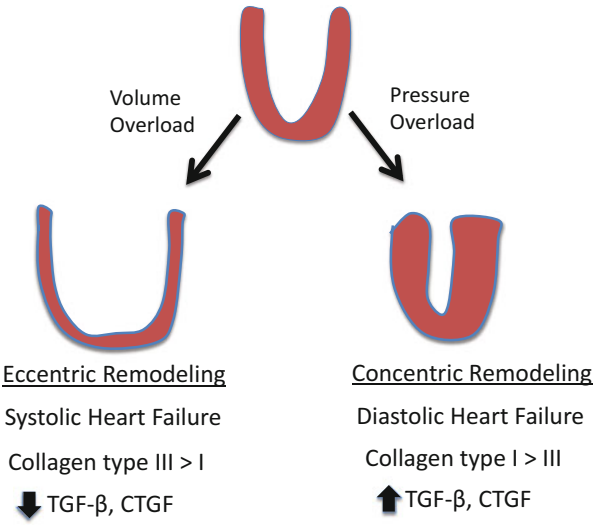


Fig. 3 Heart failure models. Conceptual differences in the clinical presentation (diastolic vs. systolic heart failure), predominant collagen type (I vs. III), and pattern of expression of regulatory factors, based on studies from animal models

the ventricular walls thicken and stiffen. Both concentric and eccentric remodeling can be induced in animals through genetic manipulation, infusion of chemicals, or surgery. For example, eccentric remodeling can be induced through systems of *volume overload*, in which a connection is created between the aorta and the vena cava allowing large amounts of blood to flow through the heart. To model concentric remodeling, investigators have used models of *pressure overload*, in which a band is placed across the aorta, inhibiting outflow from the ventricle into the body (See Fig. 3).

In models of volume overload, there appears to be increased turnover of ECM (Hutchinson et al. 2010), collagen isotype changing from type I to III (Hutchinson et al. 2010), and downregulation of TGF-β and CTGF (Zheng et al. 2009). In contrast, models of pressure overload have shown increased deposition of both types of collagen (I and III), as well as fibronectin (Grimm et al. 2001) and an increase in inhibitors of metalloproteinases (TIMPs) (Rysa et al. 2005). In addition, TGF-β signaling (Grimm et al. 2001) and CTGF and osteopontin (Xie et al. 2004) are increased. Generally, changes in collagen I contribute more to diastolic dysfunction due to its increased stiffness (Zile and Baicu 2013), and so one might also anticipate an increase in type I over type III collagen in individuals with diastolic heart failure.

To move from the bench to interpretation of clinical studies, one must understand the methods used to quantify heart failure clinically. Heart failure is typically categorized based on the presence of symptoms (shortness of breath, leg edema, elevated neck veins, 6 min walk distance) and the ejection fraction of the heart (the change in volume with each pump). In most of these studies, systolic heart failure is classified by an ejection fraction below 30–35 %, and diastolic heart failure is classified by the presence of heart failure symptoms in individuals with a normal (preserved) ejection fraction (>50 %). These two states are also referred to as "heart failure with reduced ejection fraction" (HFrEF) and "heart failure with preserved

ejection fraction" (HFpEF). Based on the clinical nature of these definitions, one might see where circulating biomarkers that display differential effects between systolic and diastolic heart failure (e.g., TGF-β) might not necessarily provide a consistent signal in a clinical study, despite more consistent results in an animal model. Nonetheless, in order to eventually be useful clinically, a given biomarker will need to provide enough "signal" to exceed the clinical "noise," a goal that to date no fibrosis biomarkers have clearly and consistently shown.

A final critical point to consider in studies of ventricular fibrosis concerns the relatively recent developments in cardiac imaging to allow visualization of larger areas of scar in the ventricle using late gadolinium enhancement (LGE). The use of LGE to quantify ventricular fibrosis has allowed investigators to better detect and quantify this process in clinical populations. While this approach is beginning to be incorporated into clinical care, there are still reasons that circulating markers of fibrosis would provide important information. For one, circulating markers are likely to be released prior to the onset of frank scar formation, and thus early detection might be useful to prevent future scar formation. Second, although fibrosis might be present on cardiac MRI, this finding alone does not provide information about the pathways leading to development of fibrosis, which could be targeted therapeutically. Finally, many individuals in whom information about ventricular fibrosis would be useful are unable to undergo cardiac MRI imaging due to the presence of implantable cardioverter defibrillators (ICDs) (see section "Cardiac Fibrosis Biomarkers in Ventricular Conditions: Arrhythmias and Sudden Cardiac Death").

To focus thinking, we have grouped the studies of circulating fibrosis biomarkers and heart failure by outcome, namely, whether they are used to examine the onset of heart failure in those without (incident heart failure) or to predict mortality or worsening of symptoms in individuals already diagnosed with heart failure (prevalent heart failure). A collection of these studies is presented in Table 1.

Incident Systolic Heart Failure

To date, there have not been large, population studies demonstrating that fibrosis on cardiac MRI is a risk factor for incident heart failure. One study of 187 diabetic patients showed that fibrosis was associated with a threefold increase in risk of the combined endpoint of major cardiac events that included new heart failure (Kwong et al. 2008). It seems feasible that a method to detect fibrosis prior to the onset of heart failure symptoms would be beneficial as well, which makes detection of lower levels using circulating fibrosis biomarkers particularly appealing in this process.

Longitudinal cohorts that store blood samples are convenient sources to examine predictors of incident heart failure, and several longitudinal studies have demonstrated a role of measurement of circulating fibrosis biomarkers on risk. Barasch and colleagues examined multiple circulating fibrosis biomarkers in a large cohort of elderly individuals and found that CITP and PIIINP were associated with development of both systolic and diastolic heart failure (Barasch et al. 2009). There was no significant association of PICP with heart failure, and in general, fibrosis markers did not significantly differ between diastolic and systolic heart failure, whereas NT-proBNP values were higher in systolic heart failure than in diastolic heart failure

Table 1 Clinical studies of circulating fibrosis biomarkers and heart failure

	Biomarker	Association	Citation
Incident heart failure	Gal-3	Increased LV mass, heart failure, and all-cause mortality	(Ho et al. 2012)
	PICP, PIIINP, CITP	CITP and PIIINP associated with increased risk of SHF and DHF; PICP not associated	(Barasch et al. 2009)
	TGF-β	Increased heart failure	(Glazer et al. 2012)
Prevalent systolic heart failure	PIIINP, MMP-1	Inverse association with 6-min walk test and survival	(Radauceanu et al. 2008)
	PIIINP	Inverse association with death	(Zannad et al. 2000)
	PIIINP	Increased hospitalization and mortality	(Cicoira et al. 2004)
	MMP-1, TIMP-1	MMP-9 no effect, TIMP-1 increased with death	(Frantz et al. 2008)
	MMP-2, MMP-3, MMP-9, TIMP-1	MMP-2, MMP-9, and TIMP-1 increased in heart failure; MMP-3 no association	(George et al. 2005)
	PIIINP, ICTP	Decreased survival	(Klappacher et al. 1995)
	PIIINP	Increased risk of cardiac events	(Sato et al. 1997)
	PIIINP, PINP, PCIP, MMP-1, TIMP-1	PIIINP increased in heart failure, MMP-1 lower in heart failure	(Alla et al. 2006)
Prevalent diastolic heart failure	TIMP-1	Increased with heart failure	(Ahmed et al. 2006)
	PICP, PIIINP, PINP, CITP, MMP-2, MMP-9	Increased with heart failure	(Martos et al. 2007, 2009)
	sST2	Increased mortality and heart failure events	(Rehman et al. 2008; Manzano-Fernandez et al. 2011; Wang et al. 2013)
	PICP, PINP, PIIINP, CITP, MMP-1, MMP-2, MMP-9, TIMP-1	PIIINP, CITP, TIMP-1, active MMP-2 and active MMP-9 all associated with DD; PINP, PICP, MMP-1 no association	(Lombardi et al. 2003)

(Barasch et al. 2009). This single study suggests that increased production of collagen III and turnover of collagen I are predictive equally between diastolic and systolic heart failure, although the lack of association of PICP, particularly in diastolic heart failure, is more challenging to explain. Ho et al. examined galectin-3 in 3,353 participants of the Framingham Offspring study and found that increased levels were associated with increased ventricular mass, incidence of heart failure, and all-cause mortality (Ho et al. 2012), even after adjustment for NT-proBNP levels and other clinical risk factors. This study is the largest to date and is one of several (see later) to suggest galectin-3 might be independently useful to study cardiac fibrosis for both incident and prevalent heart failure. Glazer et al. reported that TGF-β was associated with increased risk of incident heart failure from a nested

case-control study (Glazer et al. 2012), which also supports a role for TGF-β in detection of fibrosis, although as will be discussed later, this factor has shown less consistent results than others in clinical studies.

Prevalent Systolic Heart Failure

In prevalent heart failure populations, fibrosis seen on cardiac MRI has been associated with increased adverse outcomes (Gulati et al. 2013), providing some background for the use of circulating fibrosis biomarkers (Table 1). The most consistent finding appears to be that of PIIINP, which across studies is consistently associated with adverse events in patients with systolic heart failure (Cicoira et al. 2004; Radauceanu et al. 2008; Klappacher et al. 1995; Zannad et al. 2000; Sato et al. 1997; Lopez-Andres et al. 2012). Kaye et al., despite issues with specificity to cardiac release issues discussed above, did find that PIIINP was closely correlated with the pulmonary capillary wedge pressure and the estimated glomerular filtration rate (Kaye et al. 2013). TIMP-1 levels have also been associated with adverse events, although for many MMP and related factors, the results are somewhat mixed (Alla et al. 2006; George et al. 2005; Frantz et al. 2008). Schwartzkopff examined a number of fibrosis biomarkers in a small number of patients with systolic heart failure compared with controls and found that the MMP/TIMP ratio and levels of CITP, but not PICP, were associated with systolic heart failure (Schwartzkopff et al. 2002).

These studies are consistent with the concept that an increase in type III collagen production (PIIINP) and overall more collagen remodeling are seen in systolic heart failure, where volume overload models would be the most applicable. Thus far, PINP has not been as useful as a marker of collagen metabolism, with no associations found in heart failure or other cardiac conditions (Alla et al. 2006; Lombardi et al. 2003; Martos et al. 2007). Investigators have speculated on a number of reasons for this, including the delayed and incomplete release of PINP from procollagen compared with PICP, as well as the possibility of degradation (de Jong et al. 2011); nonetheless, it provides an example the limitations with choosing biomarkers based on biological indications alone.

Incident Diastolic Heart Failure

There have been even fewer studies for incident diastolic heart failure, with the largest to date by Barasch et al. essentially finding the same markers to be predictive (CITP and PIIINP) in diastolic heart failure as in systolic (Barasch et al. 2009). In a meta-analysis of studies of hypertensive patients performed by Marchesi et al., MMP-9 and TIMP-1 were both elevated in hypertensives compared to controls, and MMP-2 levels were higher in hypertensives with diastolic heart failure than hypertensives without (Marchesi et al. 2012). These studies suggest a signal toward more “activity” in fibrosis pathways in patients at risk for diastolic heart failure, although clearly more work needs to be done to elicit the specific patterns and mechanisms. In addition, certain fibrosis markers have also been shown to be associated with risk factors, such as PIIINP, which was noted to be related to BMI and age in the Framingham Heart Study cohort, but not any cardiac indices (Wang et al. 2007).

Prevalent Diastolic Heart Failure

In patients with aortic stenosis and diastolic heart failure, development of fibrosis on cardiac MRI is associated with worsened 5-year mortality (Azevedo et al. 2010), and in patients with prevalent diastolic heart failure, elevation is noted in levels of most measured circulating fibrosis biomarkers (Table 1) (Ahmed et al. 2006; Martos et al. 2007; Lombardi et al. 2003). This finding is consistent with what is seen in pressure overload models described above, with an overall increase in the deposition of collagen. Many of these studies focused on hypertensives, with comparisons between those with and without heart failure (Querejeta et al. 2004), as well as associations with filling pressures (Gonzalez et al. 2010). Zile and colleagues composed a profile panel of 17 biomarkers that they demonstrated to predict ventricular hypertrophy and diastolic heart failure in individuals with hypertension (Zile et al. 2011). In their study, they noted that an increase in MMP-2 and MMP-7 levels and decrease in MMP-8 were linked with the development of heart failure, which they attributed to resulting in greater collagen deposition.

Within specific subgroups of diastolic heart failure, the correlation between fibrosis biomarkers and fibrosis has also been studied, with mixed results. Ellims et al. examined patients with HCM to correlate imaging and serum fibrosis markers and found that regardless of myocardial fibrosis seen on cardiac MRI in patients with HCM, peripheral levels of collagen precursors were similar compared with control subjects for PINP and PIIINP (Ellims et al. 2014). In a study of patients with Fabry's disease, which also causes a hypertrophic cardiomyopathy, Kramer et al. found that collagen biomarkers were elevated in patients compared with controls, but that the levels did not correlate with LGE amount. They also noted that the presence of fibrosis on imaging was associated with an increased risk of sudden cardiac death, although the numbers were small (five patients) (Kramer et al. 2014). Ho and colleagues compared patients with pathogenic sarcomere mutations and overt hypertrophic cardiomyopathy with those who had mutations but no left ventricular hypertrophy and controls who did not have mutations and found that levels of PICP were significantly higher in mutation carriers without left ventricular hypertrophy and in subjects with overt hypertrophic cardiomyopathy than in controls. In their study, the ratio of PICP to CITP was increased only in subjects with overt hypertrophic cardiomyopathy, providing further support for the idea that collagen deposition was leading to the hypertrophy that developed. They also performed cardiac MRI studies that showed myocardial fibrosis only in those subjects with overt hypertrophic cardiomyopathy but in none of the mutation carriers without left ventricular hypertrophy (Ho et al. 2010). Broadly, it is difficult to generalize the results from these studies to broader populations of diastolic heart failure, but they provide some outline for how comparisons between fibrosis on imaging often differ from what is inferred from sample of circulating fibrosis biomarkers.

As far as studies comparing systolic and diastolic heart failure, Lopez and colleagues examined the difference in ratio of MMP-1/TIMP-1 between systolic and diastolic HF and found that systolic HF had a larger ratio, suggesting that there was more collagen remodeling than in diastolic HF (Trueblood et al. 2001). This

study supports conclusions from animal models for systolic and diastolic heart failure, although clearly more studies are needed.

Additional Markers of Fibrosis in Heart Failure

Beyond the markers of collagen itself, a number of novel factors associated with fibrosis have been identified in populations with heart failure. Galectin-3 (mentioned above) is secreted by macrophages and increases fibroblast proliferation, activation, and transformation to myofibroblasts, as well as being linked to aldosterone signaling (Zile et al. 2004; de Boer et al. 2010). It was found to correlate with the risk of new-onset heart failure in otherwise healthy subjects (Ho et al. 2012), as well as disease severity in prevalent cases of heart failure (Motiwala et al. 2013; van Kimmenade et al. 2006). Another factor recently described has been ST2, which in the soluble form binds to the receptor for IL-33 (insoluble ST2) and prevents the anti-fibrotic signaling of IL-33 (thus, it has a net a pro-fibrosis effect). It has been examined in a number of studies, which have noted an association with worsened outcomes in individuals with prevalent heart failure (Wang et al. 2013; Manzano-Fernandez et al. 2011; Rehman et al. 2008). Gal-3 was recently studied head-to-head with soluble ST2 by Bayes-Genis and colleagues and found to contribute little to overall risk prediction after adjustment for soluble ST2 (Bayes-Genis et al. 2014).

Tromp et al. recently examined the association between syndecan-1, a heparin sulfate proteoglycan that participates in cell-ECM interactions, and adverse outcomes in both systolic and diastolic heart failure. They found a positive correlation between syndecan-1 levels and markers of fibrosis and remodeling but no correlation with inflammation markers. They also noted that there appeared to be an interaction with LV function, with a doubling of syndecan-1 associated with an increased risk of adverse outcomes in patients with diastolic HF, but not systolic HF (Tromp et al. 2014). These studies suggest that there might be a single circulating biomarker that could be sampled to identify cardiac fibrosis, although more study is needed.

As mentioned in the introduction, there are number of inflammatory markers that have been associated with heart failure, and many have been linked with ECM markers (PIIINP and IL-18; MMP-1/TIMP-1 and C-reactive protein; TIMP-1 and IL-18; MMP-1 and IL-10) (Arslan et al. 2011). Another biomarker that has been studied closely in heart failure with fibrosis markers has been brain natriuretic peptide (BNP) and its precursor, N-terminal proBNP. There is extensive literature on the associations of BNP and NT-proBNP and heart failure, which can be reviewed elsewhere. Many studies, however, include BNP or NT-proBNP in their biomarker analysis since mechanistically, adjustment for natriuretic peptide level might theoretically normalize to changes in wall stress observed in heart failure. ECM markers have also been positively associated with natriuretic peptide levels (Rysa et al. 2005), and thus adjustment might provide more specific information about fibrosis, independent of merely being a marker of heart failure in general.

One interesting application of fibrosis biomarkers has been in the response to medications. As an example, some investigators have used the association of gal-3 and aldosterone to identify a group of individuals who might respond to aldosterone

antagonists with elevated gal-3 (Zile and Baicu 2013). Investigators have also described differing response with medications like spironolactone in heart failure based on level of circulating fibrosis biomarker (Zannad et al. 2000). Interestingly, Lopez et al. demonstrated that torsemide, which is known to interfere with aldosterone secretion, caused a decrease in PICP in patients with systolic heart failure, while furosemide, which does not, had no effect on PICP (Lopez et al. 2004).

To summarize, while certain circulating fibrosis biomarkers, such as PIIINP, appear to be consistently elevated across subtypes of heart failure, for the most part, there are conflicting results about patterns of collagen metabolism inferred from peripheral sampling. Some of this conflict results from sampling only a couple of the markers rather than extensively, as well as the likely inclusion of differing fibrosis phenotypes in the same group. As more and more studies are performed, we will hopefully start to get a picture of the fibrotic pathways as measured in the peripheral circulation, as well as identify potential "magic bullet" markers of fibrosis, such as soluble ST2. In the next section, we extend the examination of fibrosis biomarkers to one of the principal outcomes of patients with heart failure, sudden cardiac death, and ventricular arrhythmias.

Cardiac Fibrosis Biomarkers in Ventricular Conditions: Arrhythmias and Sudden Cardiac Death

A major concern associated with the development of cardiomyopathy is the risk of ventricular arrhythmias, in particular sudden cardiac death (SCD) from ventricular fibrillation. The relationship between LV pump function and risk of sudden death is well documented, and current medical guidelines recommend implantation of an implantable cardioverter defibrillator (ICD) in patients with a low enough ejection fraction to prevent SCD. Although there is much to be learned about the underlying mechanisms for ventricular arrhythmias when the ejection fraction becomes depressed, there is evidence that cardiac fibrosis plays a key part. Fibrosis disrupts the normal cell coupling by gap junctions and can create areas of blocked and slowed conduction necessary for the development of cardiac arrhythmias. Animal models of cardiac fibrosis had increased risk of sudden death (Fischer et al. 2007), and in humans, imaging studies have also demonstrated that the presence of ventricular fibrosis predicts sudden cardiac death and ventricular arrhythmias. A study by Gulati of 472 patients with nonischemic cardiomyopathy demonstrated that fibrosis detected using LGE was associated with an increased risk of sudden death as well as mortality (Gulati et al. 2013). Others have observed similar risk of ventricular arrhythmias and mortality with fibrosis visualized on cardiac MRI (Masci et al. 2014).

There are a limited number of studies that have specifically examined circulating fibrosis biomarkers and risk of sudden death or ventricular arrhythmias (Table 2). Kanoupakis et al. examined risk of ICD shocks in a small number of individuals who underwent implantation for primary prevention due to depressed LV function and found that increased levels of CITP, MMP-1, and TIMP-1, but not PICP, were

Table 2 Clinical studies of circulating fibrosis biomarkers and sudden cardiac death or ventricular arrhythmias

	Biomarker	Association	Citation
Sudden cardiac death (SCD)	sST2	Associated with increased risk in heart failure patients	(Ahmad et al. 2014)
	Gal-3	Associated with increased risk in heart failure patients	(Ahmad et al. 2014)
Ventricular arrhythmias	PIIINP	Decreased in patients with VT on ICD	(Blangy et al. 2007)
	PINP	Increased in patients with VT on ICD	(Blangy et al. 2007)
	Multiple biomarkers	Increased risk of arrhythmias in Fabry's disease	(Kramer et al. 2014)
	MMP-3	Increased risk of arrhythmias in adolescents with HCM	(Zachariah et al. 2012)
	MMP-1, MMP-2, and MMP-9; TIMP-1, TIMP-2, and TIMP- 4	No association with risk of arrhythmias in adolescents with HCM	(Zachariah et al. 2012)
	TIMP-1	Increased risk of VF after myocardial infarction	(Elmas et al. 2007)
	CITP	Increased with ICD shocks	(Kanoupakis et al. 2010)
	MMP-1	Increased with ICD shocks	(Kanoupakis et al. 2010)
	TIMP-1	Increased with ICD shocks	(Kanoupakis et al. 2010)
	PICP/PIIINP ratio	Increased with ICD shocks	(Flevari et al. 2012)
	MMP-9/TIMP-1 ratio	Increased with ICD shocks	(Flevari et al. 2012)
	Individual levels of PICP, PIIINP, MMP-9, TIMP-1	No association with ICD shocks	(Flevari et al. 2012)

VT ventricular tachycardia, *VF* ventricular fibrillation, *HCM* hypertrophic cardiomyopathy, *ICD* implantable cardioverter defibrillator

associated with an increased risk of shocks (Kanoupakis et al. 2010). Flevari and colleagues performed a similar analysis in a small number of individuals with primary prevention ICDs and found that no single biomarker was predictive, but that an increased ratio of PICP to PIIINP and MMP-9 to TIMP-1 was associated with an increased risk (Flevari et al. 2012). On the other hand, Blangy et al. found an inverse correlation between PIIINP (but not PINP) and the risk of VT in ICD recipients (Blangy et al. 2007), which suggests that the ratio of collagen I/III may play a role in risk. In summation, these results suggest that increased collagen remodeling (increased CITP and decreased relative TIMP-1) might be a marker of increased arrhythmic burden, although clearly more studies are needed.

The association of ventricular arrhythmias and circulating fibrosis markers has also been examined in non-heart failure populations. Elmas et al. examined serum from individuals with VF in the setting of a myocardial infarction, and those without VF, and found increased levels of TIMP-1 (and IL-8) among those with VF (Elmas et al. 2007). Zachariah et al. examined plasma from 45 adolescents with HCM for matrix metalloproteinases (MMPs) 1, 2, 3, and 9 and tissue inhibitor of metalloproteinases (TIMPs) 1, 2, and 4 and found that MMP-3 concentration was significantly higher in the group with a history of ventricular arrhythmias (Zachariah et al. 2012).

These studies were generally small and often limited to a select few fibrosis biomarkers. Ventricular arrhythmias and sudden cardiac death themselves are rare events, and so it is difficult to determine without bias or confounding the specific role of a given factor in risk. To date, there have not been enough studies to draw any sharp conclusions regarding predictiveness of any factor or combinations in terms of arrhythmic risk alone. However, this population, in particular those in whom ICDs have been implanted to allow close follow-up of arrhythmias, is ripe for discovery and validation of circulating fibrosis biomarkers on risk of ventricular arrhythmias and SCD.

Cardiac Fibrosis Biomarkers in Atrial Fibrillation

The principal atrial condition for which cardiac fibrosis has been implicated in the pathological process is atrial fibrillation (AF). During AF, the smooth conduction of the electrical signal through the atrial tissue becomes distorted, resulting in highly disorganized electrical activity detectable on a surface electrocardiogram (ECG) as baseline fibrillation. Atrial fibrosis has been widely implicated in this process and has been demonstrated in a number of settings, from animal models (Rosenberg et al. 2012a) to human studies (Platonov et al. 2011). It was shown in a goat model to play a more important role than electrical remodeling (changes in ion channels) (Cha et al. 2004) and mice that have been genetically altered to develop fibrosis and AF (Rosenberg et al. 2012a; Xiao et al. 2004). In humans, a study of autopsy specimens revealed that atrial fibrosis was highly correlated with the presence of AF (Platonov et al. 2011) (although interestingly not with advanced age, which itself had previously been thought to be a risk factor for atrial fibrosis). These studies, and many others, have demonstrated that atrial fibrosis has a significant association with AF, which thus provides motivation for the study of circulating fibrosis biomarkers for predicting AF.

At the level of clinical investigation, AF is frequently categorized based on the pattern of recurrence (paroxysmal or persistent), the setting of presentation (spontaneously or postoperatively following cardiac surgery), and the timing relative to study (incident or prevalent). There is much debate about how each of these types differs from one another pathophysiologically, although it is generally accepted that they are distinct biological entities.

Table 3 lists some of the studies that have examined circulating fibrosis biomarkers and risk of AF based on association with incident, prevalent, or

Table 3 Clinical studies of circulating fibrosis biomarkers and risk of atrial fibrillation (AF)

	Citation	Study size	Biomarkers	Findings
Incident AF	Ho et al. (2014)	3,306 Framingham Heart Study; 250 cases of AF	Galectin-3	Associated in sex and age adjusted; not significant after adjustment for other risk factors (including heart failure)
	Huxley et al. (2013)	1,080 nested case-control from ARIC; 580 cases of incident AF	MMP-1, MMP-2, MMP-9, TIMP-1, TIMP-2, PICP	All were significant in age-, sex-, and race-adjusted models; only MMP-9 was significant after adjustment for risk factors
	Rosenberg et al. (2014)	2,935 CHS cohort; 767 cases incident AF	TGF-B1, PIIINP	PIIINP nonlinear independent association; TGF-B1 no association
Prevalent AF	Sonmez et al. (2014)	52 AF cases; 33 age-matched controls	Gal-3, MMP-9, PIIINP	Gal-3, MMP-9, PIIINP higher in AF cases; all correlated significantly with LA volume index
	Mukherjee et al. (2013)	82 AF cases undergoing electrical cardioversion	MMPs (eight types); TIMPs (four types)	MMP-9, MMP-3, and TIMP-4 all were independent predictors of time to recurrence of AF
	Okumura et al. (2011)	50 AF cases undergoing catheter ablation for AF	CITP, MMP-2, TIMP-2	CITP and MMP-2 were higher in patients with AF recurrence; only MMP-2 was independent predictor in multivariate analysis
	Kawamura et al. (2012)	142 AF cases undergoing electrical cardioversion; 54 with AF recurrence at 24 months	PIIINP, renin, aldosterone	PIIINP was higher in individuals with AF recurrence
	Behnes et al. (2011)	401 patients with CHF symptoms; 107 AF cases	TGF-B1	Lower TGF-B1 levels in patients with AF and CHF (inverse association with LA size)

(*continued*)

Table 3 (continued)

	Citation	Study size	Biomarkers	Findings
	On et al. (2009)	86 AF cases undergoing MAZE procedure; 10 AF recurrences	TGF-B1	Higher TGF-B1 levels in patients without AF at 1-year follow-up
	Kallergis et al. (2008)	70 AF cases; 20 healthy controls	PICP, CITP, MMP-1, TIMP-1	PICP, CITP, and TIMP-1 were higher in AF cases; persistent had higher PICP but not CITP and lower MMP-1 and higher TIMP-1 than paroxysmal
Postoperative AF	Swartz et al. (2012)	54 individuals without history of AF; 18 cases of AF	PICP, PIIINP	PICP and PIIINP were elevated in AF; PICP was only independently associated
	Grammer et al. (2005)	33 patients undergoing cardiac surgery; 14 with post-op AF	ACE levels, OPN, Col I, and Col III mRNA	No association with ACE or OPN; increased Col I/Col III mRNA ratio with post-op AF

postoperative AF. Not surprisingly, a number of studies have been performed to examine the association of AF and fibrosis biomarkers (some not included in the table for brevity), which includes TGF-β1 (Kim et al. 2009), TIMPs (Kim et al. 2011), PINP (Krum et al. 2011), PIIINP (Krum et al. 2011), PICP (Lofsjogard et al. 2014), CITP (Lofsjogard et al. 2014), osteopontin (Krum et al. 2011), galectin-3 (Ho et al. 2014), and metalloproteinases (Mukherjee et al. 2013). Yet, despite the number of factors studied thus far, a clear picture of the pattern of circulating fibrosis factors in AF has not yet emerged.

Among the more robust findings, PIIINP appears to have been reproducibly associated with risk of AF incidence and recurrence, with a number of studies identifying an association (Table 3). Also in support of this association have been tissue examinations, which have provided evidence of increased levels of collagen III in AF. On et al. found that patients with persistent AF had higher levels of atrial collagen III mRNA than those in sinus rhythm (On et al. 2009). This finding was supported by other investigations including Kawamura et al. (Kawamura et al. 2012), as well as Sonmez et al., who also found that PIIINP was correlated with LA volume (Sonmez et al. 2014). In addition, Rosenberg et al. also identified a nonlinear association with PIIINP and incident AF, with an increased risk up to the median levels, and then no association beyond, even after adjustment for competing risk of death (Rosenberg et al. 2014).

In contrast, other investigations have not identified this association to the same extent. Grammer et al. found that the relative level of collagen III to collagen I mRNA was lower with postoperative AF (Grammer et al. 2005), which could indicate a difference in phenotypes between postoperative and spontaneously developing AF. However, other investigations, such as that of Xu et al., have found that the collagen I/collagen III ratio (per volume fraction) was more closely aligned with the risk of AF in terms of duration and recurrence (Xu et al. 2004). These contrasting studies are only the tip of the iceberg in terms of conflicting results for circulating fibrosis biomarkers and risk of AF, as detailed below. Principally among the limitations lies the challenge of distinguishing atrial from ventricular fibrosis from using measures of circulating fibrosis biomarkers from peripheral blood sampling. There is emerging evidence that diastolic dysfunction and diastolic heart failure directly also increase the risk of AF (Rosenberg et al. 2012b; Rosenberg and Manning 2012), and thus detection of an elevated level of circulating fibrosis biomarker from diastolic heart failure may also be found to predict development of AF. Although the possibility that circulating fibrosis biomarkers from ventricular fibrosis may also be predictive of AF does not diminish their clinical utility, the specific patterns observed may confound one another depending on the degree of contribution from the atria and ventricles. This confounding is clearly evident in the conflicting patterns observed.

Persistent Versus Paroxysmal AF

In terms of the pattern of AF recurrence, several investigations have attempted to compare persistent to paroxysmal AF. Small studies have suggested greater amounts of fibrosis in persistent than paroxysmal AF (van Brakel et al. 2013; Kottkamp 2013), although these are limited by the lack of good resolution for MRI imaging methods of fibrosis assessment in the atria and limited range of patients in whom atrial appendage samples can be collected. Shimano et al. compared levels of CITP and PIIINP in paroxysmal, persistent, and control patients and found that CITP levels, but not PIIINP levels, were higher in persistent AF patients (Shimano et al. 2008). Tziakas et al. also found that patients with persistent AF had higher CITP levels than those with paroxysmal AF (and no difference in PINP level) (Tziakas et al. 2007). In contrast, Kallergis et al. found that PICP, but not CITP, levels were higher in persistent than paroxysmal (Kallergis et al. 2008), in addition to finding that MMP-1 was lower and TIMP was higher in individuals with persistent AF. If the markers were definitely atrial in origin, and reflected atrial fibrosis, one might expect persistent AF to be similar to volume overload models of heart failure, with an increase in markers of collagen remodeling (CITP, MMPs) rather than deposition (PICP, TIMPs). However, since it is likely that the circulating levels reflect ventricular fibrosis to some degree as well, it suggests that the differences between the studies are likely to be due to differences in ventricular health and fibrosis.

Incident Versus Prevalent AF

From a mechanistic standpoint, a difference in the pattern of fibrosis biomarkers between incident and prevalent AF cases would be quite interesting since it would

indicate whether atrial fibrosis was occurring as a result of AF or fibrosis was occurring first with AF as the outcome, a question that has not been resolved in basic science studies of AF. There have not been enough large studies measuring large numbers of biomarkers to draw any of these conclusions from the data; however, certain factors have emerged as being predictive. PIIINP has been associated with the risk of AF in both incident (Rosenberg et al. 2014) and prevalent AF (Kawamura et al. 2012; Sonmez et al. 2014). MMP-9 has been found to have an association in both prevalent and incident studies, with Huxley et al. finding an association in a large nested case-control study (Huxley et al. 2013) and several investigators identifying an association in prevalent cases (Sonmez et al. 2014; Mukherjee et al. 2013). One of the principal factors in fibrosis is generally considered to be TGF-β1 (Glazer et al. 2012; On et al. 2009); however, a number of investigations have found either no clear association (Rosenberg et al. 2014) or mixed results (Kim et al. 2009, 2011; Behnes et al. 2011; On et al. 2009; Ki et al. 2010). More recent studies have also identified gal-3 as a potential marker of AF risk in both prevalent (Sonmez et al. 2014) and incident (Ho et al. 2014) populations, although more studies are likely needed.

Association of AF and Heart Failure

There is a well-known association between heart failure and AF, which has significant implications when it comes to interpretation of circulating fibrosis biomarkers. As mentioned above, collagen metabolites can be released from the ventricle due to ventricular fibrosis in heart failure, or from the atria due to atrial fibrosis, which is often correlated with atrial enlargement. Few investigators have attempted to tease out these subtleties, if not by distinguishing factors specific to heart failure then at least through identification of factor-associated atrial size. Among prevalent heart failure patients, Lofsjogard found that increased PICP was associated with the presence of AF, and that CITP was associated with LA volume, but not AF (Lofsjogard et al. 2014). Kim et al. also examined for association with LA size and found that both TGF-B and TIMP-1 were associated (Kim et al. 2011). These studies are too limited to draw any conclusions; other than that, there is conflicting data, and larger studies are needed.

Recurrence of AF and Follow-Up

One approach that has been used more frequently for AF has been longitudinal measures of fibrosis biomarkers related to AF recurrence. Okumura examined levels of carboxyl-terminal telopeptide of collagen type I (CITP), metalloproteinase (MMP)-2, and tissue inhibitor of MMP-2 (TIMP-2) after ablation for AF and found that MMP-2, TIMP-2, and CITP levels had increased 2 months after ablation (Okumura et al. 2011), suggesting turnover of collagen in the heart. Kawamura examined serum PIIINP, renin, and aldosterone at 24 months in 88 patients with sinus rhythm maintenance and 54 patients with AF recurrence. They found that individuals with PIIINP levels >0.72 U/mL at baseline had significantly lower levels after 24 months than baseline (Kawamura et al. 2012).

This same group also found that candesartan significantly decreased PIIINP levels at 24 months in sinus rhythm group, but did not decrease PIIINP levels in the group with AF (Kawamura et al. 2010). These results shed some light on the effect of medications on the levels of circulating fibrosis biomarkers, similar to what was seen in heart failure. However, more studies are needed to understand the dynamic nature of these biomarkers.

To summarize, studies of circulating fibrosis biomarkers and AF have provided some evidence that markers of both collagen production (e.g., PIIINP) and collagen remodeling (MMPs and CITP) might be associated with an increased risk. More extensive work will be needed in order to determine to what extent these markers reflect atrial, rather than ventricular fibrosis, as well as the stability and consistency of the association over time. As was the case in heart failure, close examination of individual studies reveals a great deal of complexity in these associations, which range from nonlinear to confounded by other biomarker levels. Hopefully, as study sizes grow in both patients and number of biomarkers, more information will emerge about the use of circulating biomarkers of fibrosis and the risk of AF.

Limitations of Clinical Approaches

Among the key limitations to use of circulating fibrosis biomarkers, two of the biggest concerns are the need for correlation between actual cardiac fibrosis and circulating measures and the epidemiological issues of power and sample size.

There has not been a great deal of correlation attempted between actual tissue fibrosis (e.g., using cardiac MRI) and circulating fibrosis biomarkers, and to date, the results have provided highly mixed results. Querejeta et al. found that circulating levels of PICP did indeed correlate with tissue fibrosis on endomyocardial biopsy (Querejeta et al. 2000, 2004), which was confirmed using sampling from the coronary sinus to determine specificity to cardiac fibrosis. Klappacher and colleagues found that circulating PIIINP levels were highly correlated with myocardial collagen III content ($r = 0.78$), although they were also highly correlated with collagen I content (Klappacher et al. 1995). Swartz et al. found that circulating levels of PIIINP and PICP correlated with left atrial fibrosis on tissues obtained perioperatively (Swartz et al. 2012). On et al. examined atrial tissue from patients undergoing heart surgery and noted that plasma TGF-β1 levels were correlated with the degree of fibrosis (On et al. 2009). Chen et al. demonstrated that circulating PICP levels were correlated with late gadolinium enhancement in the RV of patients undergoing repair of tetralogy of Fallot (Chen et al. 2013). These studies all provide some degree of feasibility for circulating fibrosis biomarkers as indicators of actual cardiac fibrosis.

On the other hand, Kaye et al. were unable to detect cardiac release of either PINP or PIIINP in controls or HF patients using transcardiac blood sampling, despite finding increased peripheral blood levels in heart failure patients (Kaye et al. 2013). Ellims et al. were also unable to validate cardiac release of biomarkers of collagen

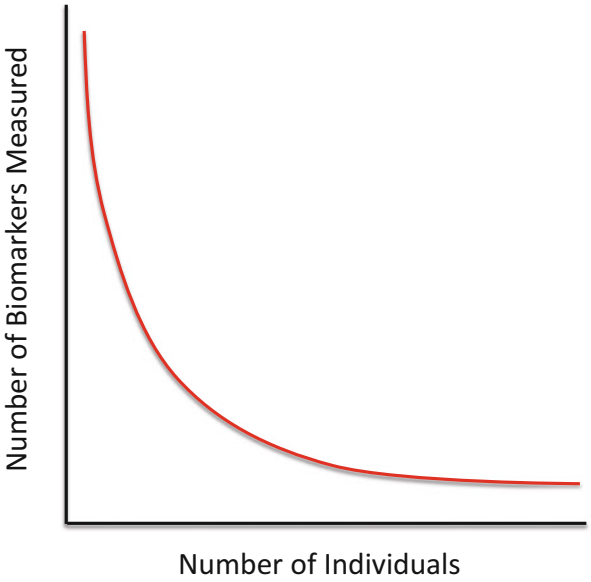


Fig. 4 Clinical studies trade-off. Conceptual diagram of the general trade-off seen with many clinical studies of circulating fibrosis biomarkers (*red line*). Most studies are limited to either a large number of factors measured in few individuals or a small number of factors measured in a large number of individuals. See text for more discussion

synthesis in hypertrophic cardiomyopathy patients (Ellims et al. 2014). Although these studies do not necessarily dispute the use of circulating fibrosis biomarkers as clinical predictors, they do indicate that more work is needed if investigators hope to eventually tie results to actual biology. It is also important to recognize that these markers are not specific to the heart, and that circulating fibrosis biomarkers have been associated with progression of liver disease, pulmonary fibrosis, and a range of other pro-fibrotic conditions.

Moving from biological issues with the use of fibrosis biomarkers to the world of epidemiology and clinical investigation also brings a number of study issues. For one, there is only limited information available about intra- and interindividual changes in biomarker levels over time for most of the markers. This information would be absolutely necessary for markers to be useful, as a biomarker that displays wide fluctuations within a given individual would be very difficult to use in the clinical decision-making process. A separate but related issue concerns study power, with most clinical studies of circulating fibrosis biomarkers falling somewhere along the spectrum of measuring a large number of biomarkers in too few individuals or measuring too few biomarkers in a large number of individuals (Fig. 4). Thus, studies tend to be too small in people to adjust for the multiple clinical confounders for each condition or too small in measured factors to account for the complex relationships between factors that are frequently part of the same pathway. The latter is a common issue in studies of circulating fibrosis biomarkers, as a "complete" study of the state of collagen metabolism should include all factors (PICP, PINP, PIINP, CITP, etc.), not simply a handful of cherry-picked ones. These limitations overall are the result of limited resources and the high costs required to study a large number of biomarkers simultaneously in a large number of individuals (preferably

also at several time points); however, they will likely need to be overcome if we hope to truly bring the bench to the bedside in using circulating fibrosis biomarkers to understand the role of cardiac fibrosis in health and disease.

Potential Applications to Prognosis, Other Diseases, or Conditions

There is a great deal of excitement about the potential uses of circulating fibrosis biomarkers in detection of cardiac fibrosis, with benefits of early detection of heart failure, and prediction of risk of atrial and ventricular arrhythmias. In addition to providing markers that can be easily and noninvasively measured at regular intervals in the management of these conditions, there is also the added potential benefit of use as treatment targets and markers of response to therapy. This potential is limited by current issues highlighted above, as well as nonspecific associations.

The potential for measurement of circulating markers of fibrosis presents a unique opportunity for translation from the bench to the bedside, as it allows measurement of factors that had previously been solely available on tissue removed from the body. However, with this potential comes a number of questions big and small about the application of research methods designed to address one or another questions. To date, no circulating fibrosis biomarker has come close to clinical use, and based on these preliminary studies, it seems likely that more flexible or broader approaches will be needed before any additional information beyond what has been learned from prior tissue studies will be gleaned from studies of circulating fibrosis biomarkers. In particular, investigators will need to decide whether the goal is to find the "magic bullet" biomarker that explains much of the risk of disease or identify biological patterns of biomarker levels, unique to different individuals that might provide information about future risk. Certain investigators have advocated the "panel" approach to measurement of biomarkers (Zile and Baicu 2013), although this is not always feasible. It will be up to future investigations to perform larger studies that include both large numbers of individuals and large numbers of biomarkers. At that point, we will hopefully learn what is necessary about how measurement of circulating fibrosis biomarkers can be performed to determine cardiovascular health.

Summary Points

- Cardiac fibrosis is closely linked with many pathological cardiac conditions, including heart failure, ventricular arrhythmias and sudden death, and atrial fibrillation.
- A number of products of collagen metabolism can be measured in the circulation as biomarkers of cardiac fibrosis.

- In heart failure, there appears to be a difference in the patterns of fibrosis and fibrotic signaling between models of systolic dysfunction (volume overload) and diastolic dysfunction (pressure overload).
- In clinical studies of systolic heart failure, markers of collagen III upregulation and collagen turnover/remodeling appear to correlate with disease severity and adverse outcomes.
- In clinical studies of diastolic heart failure, increased levels of both types of collagen have been noted, although the results are mixed in terms of specifics.
- In clinical studies of ventricular arrhythmias and sudden death, it appears that markers of collagen turnover are associated with risk, although the numbers are very small and more studies are needed.
- In atrial fibrillation, where atrial fibrosis has a strong association with disease, the results are similar to that seen in heart failure in terms of which biomarkers are association with risk, raising the important issue of what is the sources of the fibrosis that will need to be determined.
- There are several biological and epidemiological limitations to investigations of circulating fibrosis biomarkers and cardiovascular disease that will need to be overcome for these markers to be clinically useful.

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Adiponectin as Biomarker in Coronary Artery Disease

27

Sonia Eiras and José Ramón González-Juanatey

Contents

Abstract

Adiponectin is one of the main proteins produced and released by mature adipocytes. It was identified at the same time by four different groups and assigned the following names: Acrp30, adipoQ, GBP28, and apM1. This last one makes reference to the gene, which is localized in the chromosome 3q27. The adiponectin protein has four domains: a signal peptide, a variable region among species, a collagenous domain and a carboxyterminal globular domain. Usually,

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_9

the adiponectin forms dimers, trimers, or structures which are more complex. Thus, monomers are not found in plasma. Their circulating levels represent the 0.01 % of total plasma proteins. Several studies have described their anti-atherogenic and insulin sensitizer properties because patients with CAD or T2DM had low plasma levels of this protein. Moreover, a range from 3.3. to 4.2 μg/mL can determine lesion complexity in patients with ACS. These results suggest that adiponectin levels are associated with CAD and its extension or severity. Moreover, they can be a good predictor of CAD because the included patients in the CACTI study with low adiponectin levels progressed with high coronary artery calcium volume. In fact, lower levels than 4.4 μg/mL were described as predictors of higher risk of death and myocardial infarction in patients who underwent coronary angiography with stable angina. The main considered CAD risk factors such as obesity, diabetes, dyslipidemia, and hypertension were associated with low concentrations of plasma adiponectin. In this sense, loss of weight, some antidiabetic and antihypertensive drugs, and statins were found to be good inducers of an increment of plasma adiponectin levels with benefits over endothelial and muscle cells.

Keywords

Adiponectin • Coronary artery disease • Cardiovascular risk factors • Indicators • Diagnosis • Prognosis • Regulators

Abbreviations

aa	Amino acids
ACRP30	Adipocyte complement-related 30 kDa protein
ACS	Acute coronary syndrome
apM1	Adipocyte C1q and collagen domain-containing protein
BIP	Bezafibrate Infarction Prevention
BMI	Body mass index
CACTI	Coronary artery calcification in type 1 diabetes
CAD	Coronary artery disease
cDNA	Complementary deoxyribonucleic acid
GBP28	Gelatin-binding protein
GLP-1	Glucagon-like peptide-1
HDL-C	High-density lipoprotein cholesterol
LDL-C	Low-density lipoprotein cholesterol
MESA	Multi-Ethnic Study of Atherosclerosis
mRNA	Messenger ribonucleic acid
ROS	Reactive oxygen species
STEMI	ST segment elevation myocardial infarction
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TCFA	Thin-capped fibroatheroma
TG	Triglycerides
UniProt	Universal Protein Resource

Key Facts of Adipogenesis

- Process involving molecular, cytoskeletal, and functional changes to adipocyte, with spherical shape, development from preadipocytes, with fibroblast-like shape, or mesenchymal stem cells.
- Mechanism involving transcription factors, proteins that bind to DNA-specific region for increasing gene expression, such as peroxisome proliferator-activated receptor gamma.
- Differentiation of preadipocytes or mesenchymal stem cells to mature adipocytes that are able to produce and release adipokines which exert effect on several target organs for regulating appetite (leptin), insulin sensitizing (adiponectin), inflammation (resistin, chemerin), etc.
- Differentiation process in adipose tissue which is necessary for inducing adiponectin expression and secretion. This protein is an adipokine with anti-inflammatory, anti-atherogenic, and insulin sensitizer properties.

Definitions

Acute coronary syndrome Obstruction of coronary artery lumen by plaque disruption in combination of platelet aggregates, fibrin, and red blood cells. This process alters the sequence of depolarization reflected as changes in the surface of QRS. A high percentage of patients have an electrocardiogram with ST elevations because of Q wave's evolution in the leads overlying the infarct zone, but there are patients without ST elevation or unstable angina.

Coronary artery disease Obstruction of the coronary arteries by atheromatous plaque without uniform signs and symptoms. The tool for the quantification of CAD burden is the coronary angiography.

Gensini score Coronary angiographic score determined by Gensini G.G. for detecting the stenotic lesions severity of the plaque.

ST segment elevation myocardial infarction Acute coronary syndrome associated with ST elevations in the electrocardiogram.

Introduction

General Definition of Adiponectin

The catalog of information on proteins, UniProt, describes on its web page http://www.uniprot.org/uniprot/Q15848 the main characteristics of adiponectin. We can find, in the literature, alternative names as 30 kDa adipocyte complement-related protein, ACRP30, adipocyte C1q and collagen domain-containing protein, apM-1,

Fig. 1 Domains of adiponectin

or GBP28. The amount of alternative names is due to this protein was discovered by four independent groups (Scherer et al. 1995; Hu et al. 1996; Maeda et al. 1996; Nakano et al. 1996) using different methods. But, the first data were published by Philipp E. Scherer et al. on 1995 (Scherer et al. 1995) after analyzing the full-length cDNA library templated by mRNA from 3T3-L1 adipocytes at day 8 of differentiation. With these experiments they identified a specific protein of mature adipocytes, called adiponectin. This is constituted by four domains. The C-terminal globular domain contains 137 aa, the next C1q and collagen-like domain contains 65 aa, the variable domain with 28 aa, and the N-terminal and signal sequence with 17 aa (Goldstein et al. 2009) as Fig. 1 shows.

Collagen-like domain forms homo-trimers, which further combine to make oligomeric complexes. In serum, adiponectin can be found as low or high molecular weight complex. The first one is constituted by dimers or trimers. However, most of the studies showed adiponectin concentrations without considering the proportion of different complexes.

Plasma Adiponectin Levels and Coronary Artery Disease

At this moment, the inflammatory process on CAD is well known (Libby et al. 2002). Thus, the atherosclerosis progression involves inflammatory cells which release pro-inflammatory mediators (cytokines, chemokines, reactive oxygen species, and nitrogen species) (Moore et al. 2013) as protector mechanism against modified lipids or proteins by ROS (Miller et al. 2011), sugar (Price and Knight 2007), etc. In this sense, after perceiving the anti-inflammatory property of adiponectin, several groups have tried to identify the association between this protein and CAD. Table 1 summarized some findings with respect to this subject. In 2005, Kojima et al. described lower levels of adiponectin in patients with CAD (5.8 ± 3.2 vs. 9.1 ± 5.2 μg/mL). They defined CAD patients whose coronary angiography showed $\geq$50 % narrowing of the major coronaries and control group who had atypical chest pain at rest or following minimal exercise associated with coronary spasm or $\leq$25 % narrowing of the major coronaries. The exclusion criteria were thiazolidinedione treatment, symptoms of atrial fibrillation, peripheral artery diseases, or other inflammatory diseases (Kojima et al. 2005). However, in this CAD group, there was a higher proportion of patients with glucose intolerance and high levels of pulse pressure and C-reactive protein than those without CAD. After analyzing its correlation to these factors, they found a negative association with glucose intolerance and C-reactive protein. These results determined the relationship between adiponectin and CAD, glucose metabolism, and inflammation process. Although they signed differences regarding gender, Kumada et al. had described, 2 years earlier, a cutoff value (<4 μg/mL) of adiponectin for detecting CAD prevalence, in men, with independence of the other risk factors

Table 1 Adiponectin levels in CAD patients

Author	Year	Clinical patients	Adiponectin levels (μg/mL)
Dabelea D	2003	T1DM with prediction of coronary atherosclerosis	5.2
Kumada M	2003	≥75 % stenosis, men, stable CAD	<4.0 cutoff
Kojima S	2005	≥50 % stenosis, stable CAD	5.8 ± 3.2
Otsuka F	2006	ACS and single complex lesions	4.21 [3.36–5.41]
Otsuka F	2006	ACS and multiple complex lesions	3.26 [2.26–4.46]
Cavusoglu E	2006	Angina and non-STEMI with prediction of death or myocardial infarction	≤4.4
Sawada T	2008	≥75 % stenosis, men, without TCFA	10.9 ± 4.3
Sawada T	2008	≥75 % stenosis, men, with TCFA	Similar to Otsuka F

(Kumada et al. 2003). In spite of the fact that they considered CAD group whose coronary angiography narrowed ≥75 %, at least in one of the major coronary arteries, their adiponectin levels were similar to those described by Kojima et al. (2005). Two years later, (Otsuka et al. 2006) tested 207 men with CAD and determined, even, lower levels in those patients with ACS and multiple complex lesions. Thus, patients with ACS and single complex lesions had 4.21 [range 3.36–5.41] μg/mL of adiponectin and patients with multiple complex lesions contained 3.26 [range 2.26–4.46] μg/mL. These data suggested the association between low adiponectin levels and CAD extension.

Another important point is the severity of the disease which can be calculated by assigning a score to each coronary stenosis according to (a) the degree of luminal narrowing and (b) its importance due to localization, as it was described by Gensini (1983). Hence, the association between adiponectin and severity of CAD was confirmed after visualizing an inverse correlation between adiponectin levels and Gensini score (Hara et al. 2007). But the development of imaging techniques as multislice computed tomography, angiography, and virtual histology intravascular ultrasound together with optical coherence tomography (Sawada et al. 2008) let the scientific community know the vulnerable plaques and classify them according to their composition (fibrotic, fibrofatty, necrotic core, dense calcium) as fibrocalcific, fibroatheroma, and TCFA (van Velzen et al. 2009). This last plaque type identifies the patients more vulnerable with percentage of necrotic core >10, without evidence of an overlying fibrous component and percentage of plaque volume >40. This approach allowed detecting the association between adiponectin levels and TCFA presence (Sawada et al. 2008). These authors selected men patients with stable CAD, coronary stenosis ≥75 %, without occluded, highly calcified vessels, significant left main artery disease, or severe tortuous lesion. These inclusion and exclusion criteria could explain their higher levels of adiponectin in patients with stable CAD without TCFA (10.9 ± 4.3 μg/mL) than those described previously (Otsuka et al. 2006). However, the found levels in patients with multivessel TCFA were comparable to those described in patients with ACS and multiple complex lesions. The high frequency of an ACS past history in patients with TCFA might suggest the contribution of plasma adiponectin analysis for stratifying the patients with high risk.

The importance to identify targets for primary prevention of CAD determined that further analyses were carried out regarding plasma adiponectin levels. The finding of new molecules or parameters as predictors needs occasionally prospective cohort studies in progression. Like this, one of the useful studies for this analysis was the CACTI study for evaluating the development and progression of subclinical CAD in subjects with T1DM and without diabetes (Dabelea et al. 2003). In this study, the coronary artery calcium volume was collected from each patient with follow-up for 1.6–3.3 years and their plasma adiponectin levels. The next analysis determined the association between low plasma adiponectin levels and progression of calcium volume score (Maahs et al. 2005). Thus, the median range 5.2 μg/mL levels of this protein might add a new appreciated value as predictor of coronary atherosclerosis progression. Later, Cavusoglu et al. (2006) described adiponectin values $\leq$4.4 μg/mL as predictors of higher risk of death and myocardial infarction at 2 years of follow-up in patients who underwent coronary angiography with stable angina, troponin-negative unstable angina, and non-STEMI. But, also, in other prospective study, low adiponectin levels after myocardial infarction in patients with STEMI were predictors of any cause of death of patients (Lindberg et al. 2012).

So far, low adiponectin levels seem to be a good indicator of CAD, predictor of all-cause mortality in these patients, and predictor of higher risk of death and myocardial infarction in patients without CAD. In opposite, high adiponectin levels were found to be a good predictor of all-cause mortality in patients with carotid atherosclerotic disease (Persson et al. 2012). It is a paradox because both diseases are characterized by an atherosclerotic process which is associated with low adiponectin levels (Nishida et al. 2007). This situation is comparable to obesity paradox (Vemmos et al. 2011) where obese and overweight stroke patients have better early and long-term survival rates compared to those with normal BMI. Some authors explain that adiponectin might increase in response to extension of neurological damage (Kuwashiro et al. 2014); however, it should take into account the differential mechanisms between CAD and stroke. Thus, CAD is characterized by homogeneous risk factors such as obesity, diabetes, or dyslipidemia. The adiponectin levels regarding these factors are summarized in Table 2.

Plasma Adiponectin and Coronary Artery Disease Risk Factors

Obesity

Hyperplasia and hypertrophy of adipocytes are the main characteristics of adipose tissue in obese subjects (Avram et al. 2007). At this point, their adipocytes should be expressing higher levels of adiponectin because of a mature adipocyte – protein. However, the negative correlation between adiponectin plasma levels and BMI determined a paradoxical finding (Arita et al. 1999). This result was unexpected because adiponectin expression appears during the adipogenesis process (Avram et al. 2007; Fernandez-Trasancos et al. 2014) as Fig. 2 shows. After considering obese subjects with BMI major than 26.4, the authors found 8.9 $\pm$ 5.4 μg/ml of plasma adiponectin levels in healthy voluntaries with normal weight and 3.7 $\pm$ 3.2 μg/ml

Table 2 Adiponectin levels and CAD risk factors (obesity, dyslipidemia, hypertension, gender)

Author	Year	Clinical patients	Adiponectin levels (μg/mL)
Arita Y	1999	Subjects with normal weight	8.9 ± 5.4
Arita Y	1999	Obese	3.7 ± 3.2
Delporte ML	2003	Women with anorexia	16.1 ± 0.9
Delporte ML	2003	Women without anorexia	11.8 ± 0.9
Hotta K	2000	T2DM with CAD and men	4.0 ± 0.4
Hotta K	2000	T2DM with CAD and women	6.3 ± 0.4
Hotta K	2000	T2DM without CAD and men	6.6 ± 0.4
Hotta K	2000	T2DM with CAD and women	7.6 ± 0.7
Matsubara M	2002	Dyslipidemia (high TG levels)	5.9 ± 0.5
Matsubara M	2002	Low TG levels	9.2 ± 0.2
Nowak L	2005	Hypertension for 8–9 years	12.5
Nowak L	2005	Hypertension for 8–9 years with antihypertensive treatment	16.9

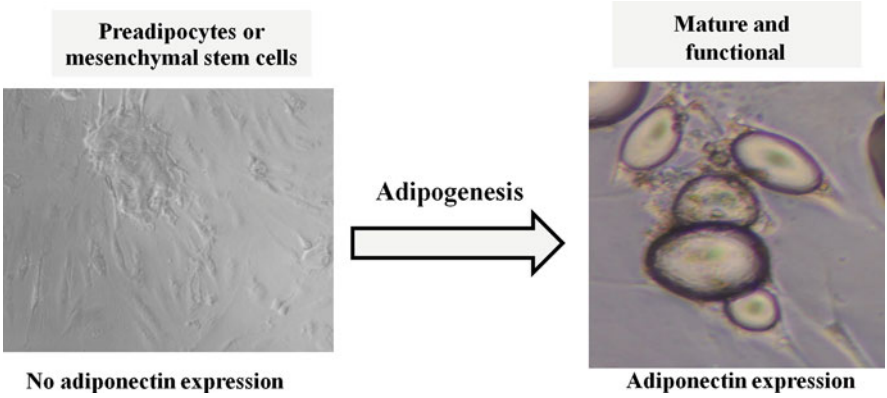

Fig. 2 Adipogenesis and adiponectin expression

in obese subjects (Arita et al. 1999). The reduction of body weight increases the adiponectin levels (Madsen et al. 2008), although it has to exceed the 10 % reduction. In fact, situations of severe weight loss, like anorexia nervosa, provoke hyperlipidemia (Delporte et al. 2003). These findings suggest that adipocytes with low hypertrophy might express more adiponectin levels. However, in familial partial lipodystrophy patients, who suffer loss of body fat after the onset of puberty, there are low levels of adiponectin (Wong et al. 2005). These patients have insulin resistance but the anorexic patients do not (Tagami et al. 2004). These findings associate the low adiponectin levels with insulin resistance. The data in anorexia nervosa can be contradictory because these patients do not have enough adipose tissue but contain high adiponectin levels. The closer explanation might be related to fasting (Kadowaki and Yamauchi

2005). Thus, after Ramadan, which is characterized by an intermittent representative fasting, the plasma adiponectin levels are augmented (Feizollahzadeh et al. 2014). However, the mechanism was not yet described. Perhaps, this adipokine is not only produced by adipose tissue, as it was found in macrophages (Luo et al. 2010) and can be regulated by self-starvation and other factors.

Diabetes

Hyperinsulinemia, associated with low glucose oxidation and energy expenditure, and hyperglycemia, associated with insulin resistance, can be regulatory factors of hypoadiponectinemia (Salmenniemi et al. 2004). These elements contribute to develop T2DM which is a disorder with atherosclerotic vascular complications (Zimmet 1992). In fact, mice with adiponectin deficiency showed mild insulin resistance and glucose intolerance (Kubota et al. 2002). In this sense, hypoadiponectinemia has been determined as a good predictor marker of diabetes development in the included patients, with fasting glucose levels between 100 and 125 mg/dL, of BIP study (Knobler et al. 2006). The classification of patients with diabetes, regarding CAD presence or absence, has determined lower levels of plasma adiponectin in patients with diabetes and CAD than those diabetic patients without CAD. The first group of patients had 4.0 ± 0.4 μg/mL in men and 6.3 ± 0.4 μg/mL in women of plasma adiponectin, and in the second group, the patients without CAD had 6.6 ± 0.4 μg/mL in men and 7.6 ± 0.7 μg/mL in women. Although plasma levels of adiponectin are higher in women than in men, there was, in both, an increase when they have neither diabetes nor CAD (Hotta et al. 2000). This association was found in different ethnic groups, (Daimon et al. 2003; Snehalatha et al. 2003) for example, Pima Indians (Weyer et al. 2001), who have tendency to be obese with T2DM. In general, the diabetic and CAD patients have low levels of adiponectin which can be playing a protector role. The next step of the scientific community was to determine the ability of diabetic treatment of increased adiponectin levels in these patients. Thus, several clinical trials with glucose-lowering agents have determined that thiazolidinedione (synthetic PPAR-γ ligands that regulate adipocyte differentiation and its endocrine function (Saltiel 1996)) treatment alone (Bailey 2005) or in combination with fibrates (Boden et al. 2007) (synthetic PPAR-α ligands that regulate lipid metabolism (Fruchart et al. 2001)) can regulate the increase of plasma adiponectin levels. These antidiabetic drugs involve nuclear receptors related with preadipocyte differentiation. In this way, their role on adiponectin regulation is comprehensible. Other and new class of antidiabetic drugs is based on GLP-1, hormone secreted by intestinal L-cells that stimulates insulin secretion. The GLP-1-related drugs also increased circulating adiponectin levels, but the mechanism is still unknown (Hibuse et al. 2014). However, the increment of insulin secretion by these drugs can improve the glucose uptake by adipocytes and induce their adiponectin production.

Dyslipidemia

The interaction between coronaries calcification and dyslipidemia regarding cardiovascular events was determined in the MESA study (Martin et al. 2014). Although

dyslipidemia and insulin resistance go hand in hand, the Japanese study, described by Matsubara et al. (2002) where diabetic patients were excluded, determined a negative correlation between plasma adiponectin levels and serum TG after adjusting for BMI. Contrary, adiponectin levels were positively correlated with serum high-density lipoprotein cholesterol (HDL-C). Thus, those patients with the highest tertile of TG contained 5.9 ± 0.5 μg/mL of adiponectin and patients with lowest tertile of TG had 9.2 ± 0.2 μg/mL of adiponectin. Moreover, low levels in patients with familial hypercholesterolemia, who contain high plasma concentrations of LDL-C serum levels, increase their risk of premature CAD (Bouhali et al. 2008). Statins, drugs for inhibiting hydroxymethylglutaryl-CoA reductase and changing the lipid profile, can be able or not to modify the serum adiponectin levels. Accordingly, the treatment with pravastatin for 16 weeks in female diabetic patients with hypercholesterolemia reduced the LDL cholesterol levels but was not able to increase adiponectin levels (Kim et al. 2013). However, in patients with isolated hypercholesterolemia, pravastatin with valsartan (angiotensin II type 1 receptor blocker) (Koh et al. 2013) or thiazolidinedione (activator of peroxisome proliferator-activated receptors) (Nezu et al. 2010) treatment decreased serum levels of LDL-C and augmented adiponectin concentration. The differential activity of pravastatin and simvastatin or the inclusion criteria of trials may explain their ability to increase or not the adiponectin concentrations. Even, simvastatin (Koh et al. 2008) or rosuvastatin (Koh et al. 2011), statin that decreases LDL-C levels and insulin sensitivity, reduced the adiponectin levels. On the other hand, pitavastatin, which has the capacity moreover to regulate HDL-C (Noji et al. 2002), increased the serum adiponectin concentrations in patients with hyperlipidemia and diabetes, but not in those without diabetes (Nomura et al. 2008).

Hypertension

The endothelial dysfunction contributes to hypertension and, in consequence, to atherosclerosis and CAD. One method for analyzing the endothelial function consists in the measure of vasodilation ability in response to reactive hyperemia after sublingual administration of nitroglycerin by strain-gauge plethysmography (Sanada et al. 2001; Ouchi et al. 2003). Thus, in patients with hypertension, defined as systolic blood pressure ≥140 mmHg and diastolic blood pressure ≥90 mmHg, but without antihypertensive treatment, the adiponectin plasma levels were associated with vasodilator response to reactive hyperemia (Ouchi et al. 2003). The conclusion is that low adiponectin plasma levels indicate an endothelial dysfunction and can contribute to clinical course of essential hypertension because their levels are associated with mean, diastolic, and systolic blood pressure (Adamczak et al. 2003). Actually, the administrated treatment against hypertension is focused on sympathetic or renin-angiotensin-aldosterone pathways and calcium channel blockers.

The reduction of sympathetic overactivity by the antihypertensive increased the adiponectin levels from 12.5 to 16.9 μg/mL in patients 25–53 aging with essential hypertension for 8–9 years (Nowak et al. 2005). In detail, although this sympathetic inhibition did not modify fat mass, BMI or insulin resistance suggests other regulator

mechanism of adiponectin levels. Again and most recent data showed that antihypertensive treatments that interrupt the angiotensin activity can also increase the adiponectin levels (Koh et al. 2007) in an independent manner of adiposity. Finally, although there was a controversy finding about the increase of adiponectin by calcium channel blockers, (Watanabe et al. 2006; Koh et al. 2009) without BMI modification, the data bear out that treatments improve the adiponectin levels in patients with hypertension, one of the CAD risk factors.

Adiponectin Expression on Coronaries

Over the coronaries exists an adipose tissue which can cover almost the 80 % of myocardium and constitute the 20 % of total heart weight (Rabkin 2007). This fat pad is named epicardial adipose tissue (EAT). Its thickness was measured by different image techniques and associated with CAD and its severity (Iacobellis et al. 2005a; Ahn et al. 2008; Gorter et al. 2008; Eroglu et al. 2009; Bettencourt et al. 2011). In spite of high EAT amount, patients with CAD expressed 40 % less mRNA adiponectin than those without CAD (Iacobellis et al. 2005b). Indeed, the protein concentration was even lower than mRNA levels. Thus, 1 g of EAT from patients without CAD had a 93 % more of adiponectin than those with CAD (Cheng et al. 2008). But, the adiponectin mRNA expression was dependent on CAD extension because it tended to fall as the number of injured coronary arteries increased (Eiras et al. 2008). In opposite, there were high concentrations of inflammatory adipokines or cytokines (Mazurek et al. 2003; Hirata et al. 2011; Zhou et al. 2011). The inflammatory process might reduce the ability of preadipocyte differentiation and, in consequence, adiponectin expression (Fernandez-Trasancos et al. 2014) as it is shown in Fig. 3. Once more, in this fat tissue, adiponectin was a good indicator of CAD but, also, of cardiovascular prognosis (Teijeira-Fernandez et al. 2012). Similar to plasma levels, adiponectin expression was also lower in patients with hypertension (Teijeira-Fernandez et al. 2008) and metabolic syndrome (Teijeira-Fernandez et al. 2011) but not in those with T2DM (Teijeira-Fernandez et al. 2010). In these last patients, some contra-regulatory mechanism might explain the differential behavior between EAT and plasma levels of adiponectin.

Adiponectin Effects on Coronaries

The atherosclerosis is an inflammatory process (Libby et al. 2002) where endothelial dysfunction promotes the induction of adhesion molecules for attaching monocytes. These cells are infiltrated into the intravascular layer and form macrophages, platelet degranulation, thrombosis, and vascular smooth muscle cell migration and proliferation (Badimon et al. 2009). The benefit effect of adiponectin between 5 and 25 μg/mL was demonstrated in human aortic endothelial cells after analyzing a reduction of TNF-α-induced monocyte adhesion (Ouchi et al. 1999) and tissue factor (Chen et al. 2008), which contributes to thrombus formation. Moreover, adiponectin

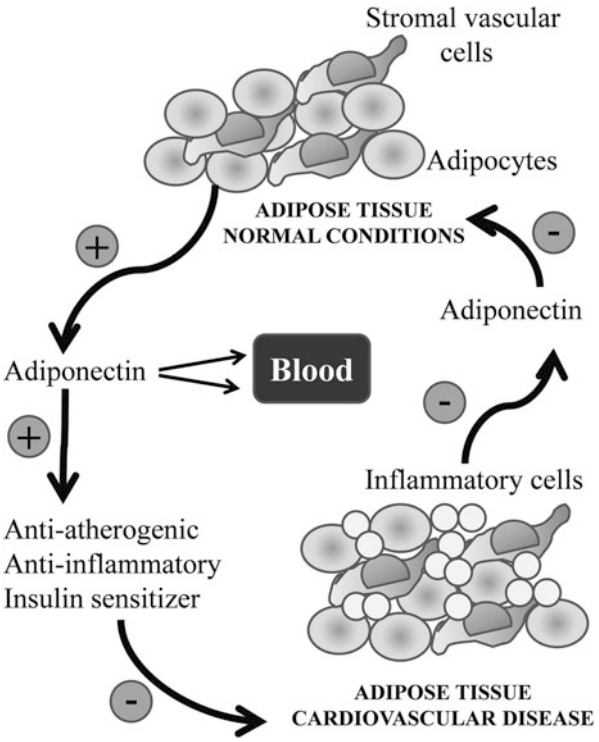

Fig. 3 Inflammation in cardiovascular disease and adiponectin regulation

induces the phosphorylation of endothelial nitric oxide synthase (Zhao et al. 2013) that produces nitric oxide for modulating vascular dilator tone and normal endothelial function. The protector effect of adiponectin was also established in smooth muscle cells where their contractile proteins and function were regulated (Ding et al. 2011).

Adiponectin Regulators

The activation of several transcription factors, PPARγ, PPARα, c/EBPβ, etc., is able to induce the adiponectin transcription. Several adipogenic drugs and hormones could increase the adiponectin expression as it is shown in Fig. 4. One adipogenic drug that regulates PPARγ is glitazone (Stumvoll and Haring 2002). This drug was a novel treatment for type 2 diabetes but its use was restricted in patients with coronary artery disease. Other adipogenic drugs are the fibrates, cardioprotectors in subjects with dyslipidemia, which are able to induce the adiponectin expression through PPARα (Sahebkar and Watts 2013). But not only drugs are good inducers of adipogenesis; there are several hormones that are able to regulate this process and also adiponectin expression. However, not all adipogenesis inducers are able to increase its expression. Thus, while growth hormone (GH), obestatin, and insulin

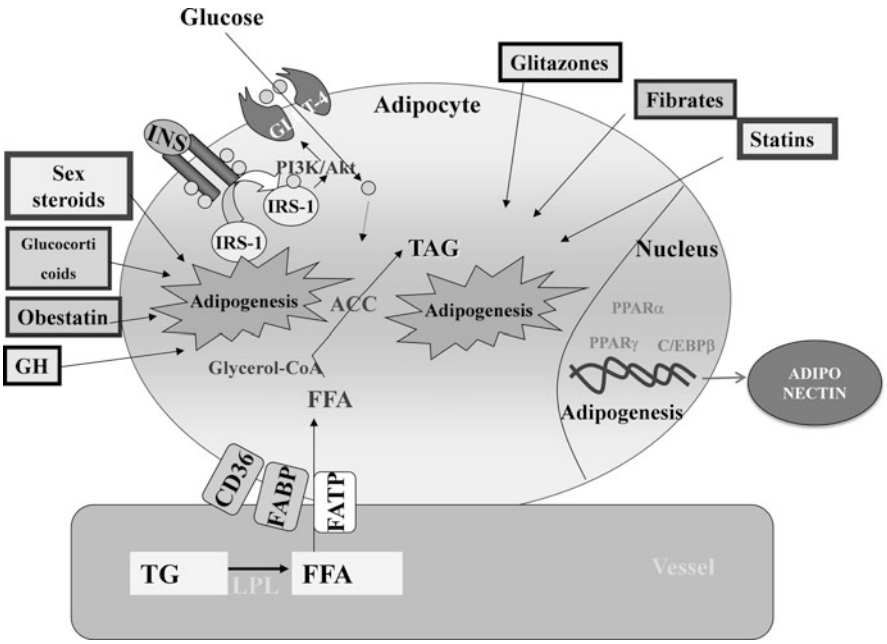

Fig. 4 Inducers of adipogenesis and adiponectin expression. *INS* insulin, *IRS-1*, insulin receptor substrate-1, *GH* growth hormone, *TAG* triacylglycerol, *ACC* acetyl-CoA carboxylase, *LPL* lipoprotein lipase, *FFA* free fatty acid, *FABP* fatty acid-binding protein, *FATP* fatty acid transporter protein

are all adipogenic hormones, insulin was not a good inducer of adiponectin expression (Xu et al. 2004; Granata et al. 2012; Koistinen et al. 2004). In fact, the adipogenic pathway might increase the adiponectin levels and prevent the coronary atherosclerosis progression. However, the rise of adipogenesis develops hypertrophic adipocytes with an inflammatory autocrine/paracrine and deleterious endocrine role. Accordingly, another pathway with a benefit on adipose tissue is the anti-inflammatory since simvastatin, pioglitazone, or their combination reduces the IL-6 expression in EAT and increases adiponectin (Grosso et al. 2014).

Potential Applications to Prognosis, Other Diseases, or Conditions

After analyzing the presented data by several authors, plasma adiponectin levels lower than 5.2 μg/mL might be considered as predictors of coronary atherosclerosis progression. However, in patients with stable angina, the limit range has to decrease because 4.4 μg/mL levels were good predictors of higher risk of death and myocardial infarction at 2 years of follow-up. Thus, adiponectin is a good indicator of CAD but, also, of cardiovascular prognosis. However, these levels do not have to be extrapolating to other atherosclerotic disorders like carotid atherosclerotic disease

because, in this situation, low adiponectin levels have a good prognosis. Moreover, because epicardial fat is the adipose tissue closer to myocardium and coronaries and its adiponectin expression is lower in patients with CAD, it might be considered as a future therapeutic target.

Summary Points

- Adiponectin is one of the main proteins produced by mature and functional adipocytes with anti-atherogenic and anti-inflammatory properties.
- Lower levels than 5.2 μg/mL of adiponectin are a good predictor of coronary artery disease.
- Lower levels than 4.4 μg/mL of adiponectin are associated with coronary artery disease.
- The adipose tissue around the coronaries, called epicardial adipose tissue, is a producer of adiponectin. But, their levels are also lower in patients with coronary artery disease.
- The epicardial adipose tissue from patients with coronary artery disease shows an increase in the inflammatory cell infiltration and inflammatory cytokines which might be decreasing the adiponectin expression.
- Hormones and drugs with effects on adiponectin upregulation might be useful as future coronary artery disease therapies.

Acknowledgments This work was funded by the project (PI13/01852), Plan Estatal de I+D+I 2013-2016 and cofunding by ISCIII-Subdirección General de Evaluación y Fomento de la investigación el Fondo Europeo de Desarrollo Regional (FEDER).

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Lipids and Lipoproteins as Biomarkers of Vascular Complications in Diabetes and Their Modulation by Dietary Phytochemicals

28

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Contents

Abstract

Lipids and lipoproteins are predictive biomarkers of vascular events in diabetes and play a critical role in the pathogenesis of macro- and microvascular complications associated with this condition. Diabetic dyslipidemia is principally

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_49

characterized by quantitative and qualitative lipid abnormalities, such as elevated fasting and postprandial triglycerides, increased production of VLDL and chylomicrons, increased glycation and oxidation of LDL and production of small dense LDL particles that are taken up by macrophages, and low HDL-cholesterol (HDL-C) and increased triglyceride content of HDL. Plant-based diets and dietary phytochemical-containing functional foods and beverages and herbs and spices have been shown to reduce LDL-cholesterol (LDL-C) and triglycerides and/or increase HDL-C in clinical studies of patients with type 2 diabetes. Plant-based diets, especially the Mediterranean diet rich in monounsaturated fats, fiber, and polyphenols, functional foods and beverages such as berries, cocoa, pomegranates, soy, and tea rich in several classes of phytochemicals and soluble fiber, and spices such as cinnamon have been shown to improve atherogenic lipid profiles in clinical studies and thus deserve special attention in the nutritional management of diabetic dyslipidemia. These phytochemical-containing diets and functional foods and beverages have been shown to modulate many pathways of lipid and lipoprotein metabolism, such as inhibiting hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting step in cholesterol synthesis, inhibiting cholesterol absorption and chylomicron synthesis, inhibiting action of fat digestive enzymes, and via improving glycemic control in diabetes. These clinical observations need further research in larger studies of patients with diabetic vascular complications.

Keywords

LDL-cholesterol • HDL-cholesterol • Type 2 diabetes • Phytochemicals • Polyphenols • Fiber • Mediterranean diet • Soy • Tea • Cinnamon

Abbreviations

ADA	American Diabetes Association
AER	Albumin excretion rate
AHEI	Alternate healthy eating index
ApoB	Apolipoprotein B
BMI	Body mass index
CAD	Coronary artery disease
CAM	Complementary and alternative medicine
CETP	Cholesteryl ester transfer protein
CHD	Coronary heart disease
CVD	Cardiovascular disease
DASH	Dietary Approaches to Stop Hypertension
FDA	Food and Drug Administration (US)
GI	Glycemic index
GSE	Grape seed extracts
GTE	Green tea extracts
HDL-C	High-density lipoprotein cholesterol
HGI	High glycemic index
HL	Hepatic lipase

HMG-CoA	Hydroxymethylglutaryl-coenzyme A
HOMA-IR	Homeostatic model assessment of insulin resistance
IMT	Intima-media thickness
LDL-C	Low-density lipoprotein cholesterol
LGI	Low glycemic index
LPL	Lipoprotein lipase
MPD	Modified prudent diet
MVC	Microvascular complications
NMR	Nuclear magnetic resonance
NPDR	Nonproliferative diabetic retinopathy
PDR	Proliferative diabetic retinopathy
PON1	Paraoxonase 1
RCT	Randomized controlled trial
RYR	Red yeast rice
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TG	Triglycerides
WPJ	Wonderful variety pomegranate juice
WPOMxl	Wonderful variety pomegranate polyphenol extract

Key Facts Related to the Role of Lipids and Lipoproteins in Diabetic Vascular Complications

- Diabetes is associated with elevated blood lipids, such as total and LDL-C and triglycerides and low HDL-C, that increase risks of blood vessel diseases mainly affecting the heart, eyes, and kidneys.
- Detailed LDL subclasses such as small LDL particles have been positively associated with risks of heart attack and eye and kidney dysfunction in diabetes.
- The American Diabetes Association recommends treatment aimed at lowering LDL-C to less than 100 mg/dL and triglycerides to less than 150 mg/dL, while increasing HDL-C to greater than 40 mg/dL for men and greater than 50 mg/dL for women.
- The "lipid triad" consisting of elevated triglycerides, low HDL-C, and small dense LDL increases risks of diseases of the blood vessels.

Key Facts Regarding Plant-Based Diets and Blood Lipid Control in Diabetes

- Plant-based diets such as the higher-fiber low-fat vegetarian diet, the Mediterranean diet, the prudent diet, and the DASH diet have been shown to lower LDL-C and triglycerides and increase HDL-C in patients with type 2 diabetes.
- Among plant-based diets, the Mediterranean diet deserved special attention in increasing HDL-C in adults with diabetes and other cardiovascular risk factors.

- These diets consist of large amounts of plant-based food groups including fruits and vegetables, whole grains, legumes and nuts, and olive oil and low amounts of meats and processed foods.
- These diets are thus high in fiber and complex carbohydrates, low in saturated fats, sugar, and sodium, and high in different varieties of phytochemicals that contribute to the lipid-lowering effects.

Key Facts Regarding Functional Foods and Blood Lipid Control in Diabetes

- Phytochemical-containing functional foods and beverages such as cocoa, fruits (berries and pomegranates), soy, and tea have been shown to lower LDL-C and/or increase HDL-C in clinical studies of patients with type 2 diabetes.
- The phytochemical content, soluble fiber, and antioxidants in these foods and beverages have been shown to cause the lowering of blood lipids.
- Dietary recommendations can be supported by health claims on these specific foods, such as consuming 25 g soy, 10 g almonds, or 2 g phytosterols on a daily basis may lead to cholesterol-lowering effects.
- Phytochemicals and soluble fiber in these foods and beverages may decrease cholesterol absorption, inhibit fat digestion enzymes, and increase the paraoxonase activity of HDL which decreases risks of diseases of blood vessels.

Key Facts Regarding Botanical Extracts and Blood Lipid Control in Diabetes

- Usage of herbs and spices for their medicinal effects is very common among patients with type 2 diabetes.
- Cinnamon has shown some effects in reducing total and LDL-C and increasing HDL-C in a pooled analysis of many clinical studies.
- Garlic and ginger extracts have shown lipid-lowering effects in patients with type 2 diabetes.
- The scientific evidence on the efficacy and safety of these herbal supplements is insufficient.

Definitions

Biomarkers Biological molecules that represent health and disease states; example, blood cholesterol

Cardiovascular disease (CVD) A class of diseases that involve the heart or blood vessels; common CVDs include: ischemic heart disease (IHD), stroke, hypertensive heart disease, rheumatic heart disease (RHD), aortic aneurysms, cardiomyopathy,

atrial fibrillation, congenital heart disease, endocarditis, and peripheral artery disease (PAD), among others

Cinnamon A spice obtained from the inner bark of several trees from the genus *Cinnamomum* that is used in both sweet and savory foods; shown to lower LDL-cholesterol and blood glucose in diabetic patients

Functional foods Foods that provide health benefits beyond basic nutrition; example, green tea

HDL Cholesterol – one of the five major groups of lipoproteins and removes and reuses LDL-cholesterol from the circulation and preventing harmful effects of LDL; elevated levels associated with reduced risk of CVD

LDL Cholesterol – one of the five major groups of lipoproteins and a common carrier of blood cholesterol typically measured in health and disease states; elevated levels associated with increased risk of CVD

Mediterranean diet Plant-based diet with high consumption of olive oil, legumes, unrefined cereals, fruits, and vegetables, moderate to high consumption of fish, moderate consumption of dairy products (mostly as cheese and yogurt), moderate wine consumption, and low consumption of meat products; popular in Greece, Italy, and Spain and shown to lower LDL-cholesterol and raise HDL-cholesterol

Polyphenols Major category of plant-based bioactive compounds in foods and beverages shown to confer protection against chronic diseases including cardiovascular disease; exert antioxidant and vasodilator actions among others; example, catechins in green tea

Triglycerides A type of lipid derived from glycerol and three fatty acids. As a blood lipid, it helps enable the bidirectional transference of adipose fat and blood glucose from the liver

Type 2 diabetes Caused by a progressive insulin secretory defect on the background of insulin resistance; diagnosis involves elevated fasting or 2-h postchallenge blood glucose and/or glycated hemoglobin (HbA1c)

Introduction

Diabetes is a metabolic condition characterized by elevated blood glucose level due to insufficient insulin production and/or peripheral tissue resistance to the action of insulin. As a significant public health problem in the USA and worldwide, diabetes increases the vulnerability for ocular, renal, neurologic, cardiovascular, peripheral vascular, and metabolic conditions, thereby increasing the risk for premature mortality,

loss of productivity, and increased medical expenditures (American Diabetes Association 2008, 2014). Cardiovascular complications are the most common macrovascular complications of diabetes, atherosclerosis being a prominent attribute considered as a still incurable disease, at least at more advanced stages (Vergès 2015). Diabetic dyslipidemia characterized by quantitative and qualitative lipid abnormalities is a major contributing factor to the elevated cardiovascular risks in diabetes (Taskinen 2003). Thus, monitoring of blood lipid levels constitutes an essential principle of the clinical care guidelines in diabetes. The American Diabetes Association (ADA) standards of care set a series of goals recommended for cardiovascular disease (CVD) prevention in type 2 diabetes, specifically involving aggressive lowering of LDL-cholesterol (LDL-C) (<100 mg/dL) while maintaining desirable levels of triglycerides (<150 mg/dL) and HDL-cholesterol (HDL-C) (>40 mg/dL in men and >50 mg/dL in women) (American Diabetes Association 2014). Large clinical trials using drugs and lifestyle interventions involving diet and exercise have demonstrated significant improvements in atherogenic lipid profiles in diabetes, though few such studies reveal a concomitant reduction in the rate of cardiovascular events.

Dietary phytochemicals have been associated with reduced incidence of type 2 diabetes and have shown promise in the treatment of diabetic dyslipidemia and vascular complications of diabetes (van Dam et al. 2013; Wedick et al. 2012). Specific phytochemical-containing functional foods and beverages, such as cocoa, soy, and tea, as well as dietary patterns comprising of plant foods, show significant benefits in the management of diabetic dyslipidemia and hyperglycemia. This review aims to present a brief summary of the role of dietary phytochemicals in the modulation of blood lipids and lipoproteins in diabetes primarily based on findings from clinical studies.

Role of Lipids and Lipoproteins in Diabetic Vascular Complications

As illustrated in Fig. 1, diabetes is associated with lipid abnormalities that are not only quantitative but also includes qualitative and kinetic abnormalities leading to a more atherogenic lipid profile. Lipid abnormalities in diabetes may be characterized as follows: elevated fasting and postprandial triglycerides, increased production of VLDL and chylomicrons, increased glycation and oxidation of LDL and production of small dense LDL particles that are taken up by macrophages, and low HDL-C and increased triglyceride content of HDL (Vergès 2015). These lipid abnormalities arise from functional abnormalities of key enzymes and lipid transfer proteins involved in lipid metabolism, especially, diminished lipoprotein lipase (LPL) activity leading to increased VLDL production, increased hepatic lipase (HL) activity leading to catabolism of HDL, and increased cholesteryl ester transfer protein (CETP) activity thereby increasing triglyceride content of LDL and HDL (Nikkilä et al. 1977;

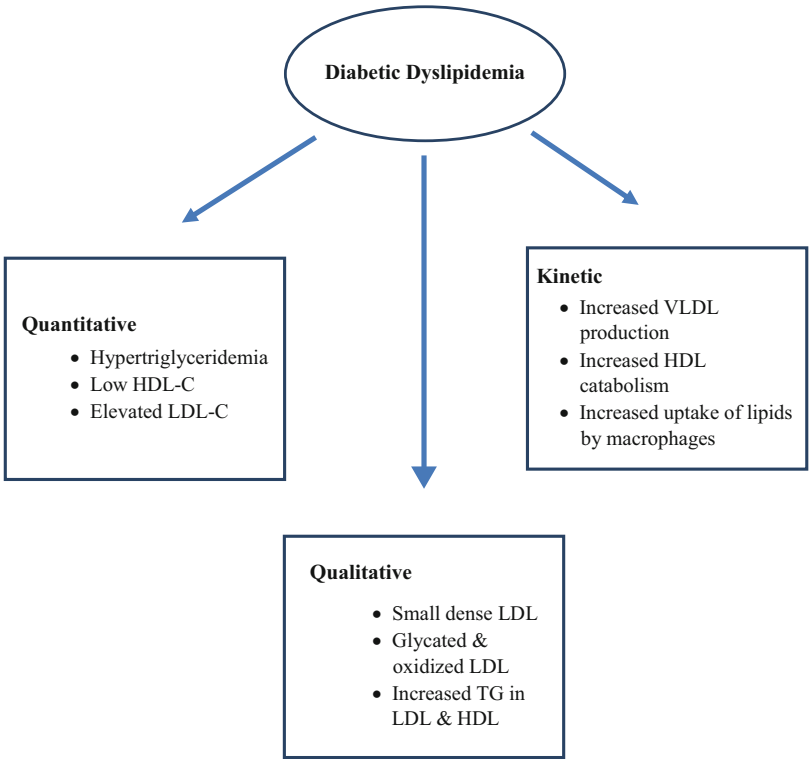

Fig. 1 Diabetic dyslipidemia and its features. The figure is a summary of the changes in blood lipids and lipoproteins in diabetes that have been associated with increased risks of macro- and microvascular complications

Bagdade et al. 1993). It has been postulated that the "lipid triad" comprised of elevated triglycerides, low HDL-C, and small dense LDL aggravates the pathogenesis of atherosclerosis in diabetes and should be the target of medical nutrition therapy (Temelkova-Kurktschiev and Hanefeld 2004). As summarized in Table 1, several lipids and lipoprotein fractions have been shown to be significantly associated with macro- and microvascular complications of diabetes. In most of these population-based studies, conventional lipids, such as LDL-C and TG, and nuclear magnetic resonance (NMR)-derived detailed lipoprotein subclasses, especially VLDL- and LDL-related particle concentrations, are positively associated with vascular complications, while HDL-C was consistently associated with reduced risks of diabetes-related cardiovascular events, retinopathy, and nephropathy. Thus, effective dietary strategies play a crucial role in the management of blood lipids, thereby leading to a more favorable lipid profile characterized by lowering

Table 1 Lipids and lipoproteins and vascular complications in diabetes: observational studies

Author, year	Study design	Subject characteristics	Significant outcomes
Soedamah-Muthu et al. (2003)	Nested case-control study	Patients with T1D ($N = 118$); diabetes duration: 15 years; age: >30 years	TG and NMR-based HDL and VLDL particle concentrations positively associated with CAD; mean LDL-C >100 mg/dL
Jenkins et al. (2003)	Cross-sectional study	Patients with T1D ($N = 968$); diabetes duration: 17 years; age: >30 years	NMR-based large, medium, and small VLDL positively associated with AER; LDL particles and ApoB with AER in men only; mean LDL-C >100 mg/dL
Lyons et al. (2006)	Cross-sectional study	Patients with T1D ($N = 968$); diabetes duration: 17 years; age: >30 years	NMR-based LDL subclasses, large VLDL, conventional LDL-C, and ApoB positively associated with carotid IMT; mean LDL-C >100 mg/dL
Zoppini et al. (2012)	Prospective study with 4.9 years follow-up	Patients with T2D ($N = 979$); diabetes duration: 14 years; age: ≥40 years	TG/HDL-C positively associated with incident retinopathy and CKD; mean LDL-C in the range of 128–132 mg/dL
Toth et al. (2012)	Retrospective study with 22 months follow-up	Patients with T2D ($N = 72{,}267$); diabetes duration: at least 1 year; age: >40 years	HDL-C inversely associated with MVC risks; LDL-C, TG, and non-HDL-C positively associated with MVC; mean LDL-C 115 mg/dL with a range of 80–150 mg/dL
Tolonen et al. (2013)	Cross-sectional study	Patients with T1D ($N = 1{,}465$); diabetes duration: 12–30 years; age: 21–51 years	Low HDL-C associated with PDR and TG associated with NPDR; non-HDL-C and ApoB associated with AER; mean LDL-C in the range of 104–132 mg/dL
Sacks et al. (2014)	Case-control study in 13 countries	Patients with T2D ($N = 2{,}535$); diabetes duration: 14 years; age: ≥40 years	Higher plasma TG and lower HDL-C associated with diabetic kidney disease in patients with LDL-C <100 mg/dL
Vazquez-Benitez et al. (2015)	Prospective study with 4.9 years follow-up	Patients with T2D ($N = 859{,}617$); diabetes duration: 15 years; age: ≥40 years	LDL-C ≥100 mg/dL positively associated with CV events and deaths

The above table is a summary of observational studies examining the associations of blood lipids and lipoproteins with macro- and microvascular complications of diabetes

LDL-C and TG and raising HDL-C. In this quest, various dietary patterns and individual foods and supplements have been identified that show good potential in blood lipid control in diabetes.

Plant-Based Diets and Blood Lipid Control in Diabetes

As summarized in Table 2, various types of plant-based diets, such as those with high fiber and low glycemic index (GI) foods, low-fat vegan and vegetarian diets, have been reported to decrease total and LDL-C and TG in adults with type 2 diabetes. In a systematic review by Ley et al. (2014), six dietary patterns were identified to be effective in the management of diabetic glycemia and subsequent reduction of cardiovascular risk factors as follows: the Mediterranean diet characterized by high consumption of minimally processed plant-based foods, olive oil as the principal source of fat, low-to-moderate consumption of dairy products, fish, and poultry, low consumption of red meat, and low-to-moderate consumption of red wine; the Dietary Approaches to Stop Hypertension (DASH) diet consisting of vegetables, fruits, low-fat dairy products, whole grains, poultry, fish, and nuts, and lower content of saturated fat, red meat, sweets, and sugar-containing beverages and sodium; the vegetarian and vegan diets that involve partial or complete exclusion of all animal-derived products; alternate healthy eating index (AHEI) dietary guidelines including greater intake of vegetables, fruits, whole grains, nuts and legumes and long-chain omega-3 fatty acids, lower intake of sugar-sweetened beverages and fruit juice, red/processed meat, trans fat, and sodium, and moderate alcohol consumption; the prudent diet characterized by higher amounts of fruits, vegetables, whole grains, legumes, and vegetable fats and lower intakes of red meats, refined grains, and sugared soft drinks; and the moderately low-carbohydrate diet that restricts consumption of carbohydrates by increasing intake of fats and proteins from animal or plant food sources (Ley et al. 2014). Interestingly, in comparison to most of the diabetic diets, the Mediterranean diet has been shown to be more effective in raising HDL-C as revealed by a systematic meta-analysis by Huo et al. (2014). The demonstrated benefits of these plant-based diets can be explained by their favorable macronutrient distribution especially the presence of monounsaturated fatty acids, high content of fiber and phytochemicals, and low amounts of cholesterol, saturated fats, sodium, and sugar that support weight loss and control of blood glucose and lipids in diabetes. With diabetes being a global epidemic, further development of region-specific dietary guidelines is needed to provide practical educational instruments, which consider variation in dietary patterns, accessibility to foods, and agriculture in different regions and cultures across the world (Ley et al. 2014; Huo et al. 2014).

Functional Foods and Blood Lipid Control in Diabetes

In recent years, researchers have focused on properties of phytochemical constituents of functional foods in the control of various aspects of diabetes; some protective effects of these compounds and food sources have been investigated in vitro and in vivo, and several clinical trials have confirmed these advantages in diabetic patients. As summarized in Table 3, popularly consumed functional foods and beverages, such as cocoa or dark chocolate, soy, tea, and fruit extracts, or a combination of functional foods have been shown to decrease total and LDL-C

Table 2 Plant-based diets and lipids in type 2 diabetes

Author, year	Study design and duration	Subject characteristics	Intervention	Effects on blood lipids	Effects on blood HbA1c, glucose, insulin, and IR
Barnard et al. (1982)	26-day program of intensive dietary Modification and exercise program; pre- and post-intervention trial	Patients with T2D ($n = 60$); mean age 61.5 ± 1.2 year; mean BW 83.2 ± 2.5 kg	Pritikin program of diet and exercise (high complex carbohydrate, high fiber, low-fat diet)	Significant decreases in serum total cholesterol and TG	Significant decrease in fasting blood glucose
Pick et al. (1996)	24-week crossover study consisting of two 12-week periods	Patients with T2D ($n = 8$); mean age 45 ± 1.5y; mean BMI 26 ± 2	High fiber, oat bran concentrate (soluble fiber [beta-glucan] content 22.8 %) bread versus white bread (control)	Significant decreases in serum total and LDL-C, and LDL: HDL	Significant decrease in blood glucose
Chandalia et al. (2000)	12-week randomized, crossover study consisting of two 6-week periods	Patients with T2D ($n = 13$); mean age 61 ± 9 y; mean BMI 32 ± 4	High-fiber diet (50 g total fiber/d) versus ADA diet (24 g total fiber/day) (control); high-fiber group had increased fruits, vegetables, and whole grains	Significant decreases in total and VLDL cholesterol and TG	Significant decreases in blood glucose and insulin
Rizkalla et al. (2004)	12-week randomized, crossover study consisting of two 4-week periods; 4-week washout phase	Patients with T2D ($n = 12$); mean age 54 ± 2 y; mean BMI 31 ± 1	LGI versus HGI carbohydrate diet; LGI diet includes whole grain cereals and beans and HGI diet includes refined cereals	Significant decreases in total and LDL-C and ApoB	Significant decreases in fasting blood glucose and insulin

(*continued*)

Table 2 (continued)

Author, year	Study design and duration	Subject characteristics	Intervention	Effects on blood lipids	Effects on blood HbA1c, glucose, insulin, and IR
Barnard et al. (2009)	74-week randomized, crossover study	Patients with T2D ($n = 99$); mean age 55 ± 9 y; mean BMI 35 ± 7	Low-fat vegan diet or conventional diabetes diet (ADA)	Significant decreases in total and LDL-C	Significant decreases in HbA1c
Kahleova et al. (2011)	24-week randomized, parallel study combining diet with or without aerobic exercise	Patients with T2D ($n = 74$); mean age 57 ± 6 y; mean BMI 35 ± 5	Vegetarian diet or control diet (conventional diabetic diet) with or without exercise training	Significant decreases in LDL-C	Significant increases in insulin sensitivity

The above table is a summary of intervention studies examining the effects of plant-based diets on blood glucose, conventional lipids, and lipoprotein subclasses in adults with type 2 diabetes

and/or increase the protective functions of HDL-C in short-term clinical studies in patients with type 2 diabetes. Meta-analyses of randomized controlled trials have demonstrated the effects of green tea, cocoa or dark chocolate, and soy in significantly decreasing total and LDL-C and/or increasing HDL-C in adults with cardiovascular risk factors (Zheng et al. 2011; Hooper et al. 2012; Anderson and Bush 2011). Green tea was shown to decrease lipid digestion and absorption in obese adults with the metabolic syndrome, thereby reducing their risks of dyslipidemia (Lisowska et al. 2015). The Food and Drug Administration (FDA) supports several health claims related to the lipid-lowering effects of functional foods as follows: 25 g soy protein in reducing LDL-C by 4 %; 10 g almonds in reducing LDL-C by 1 %; and 3.5 g β-glucan from oats, also approved for a coronary heart disease (CHD) risk reduction health claim by the FDA, can be expected to reduce LDL-C by 5 % (US FDA 2001, 2003). A heart health claim is also permitted by the FDA for plant sterols and stanols, for which the daily treatment dose is 2 g and the LDL-C reduction is 10 % (Law 2000). Soy is a complex protein with a 7 s globulin fraction to which has been attributed its cholesterol-lowering effect. This fraction may be digested to peptides with inhibitory effects on cholesterol synthesis. Another hypothesis is that the isoflavones, when linked to soy proteins, or the saponins found in soy, are also responsible for the cholesterol-lowering effect of soy (Anderson and Bush 2011). Similarly, nuts have vegetable proteins, monounsaturated fats, plant sterols, and a range of other bioactive phytochemicals and antioxidants that may have an impact on CHD risk factors (Ruiz Ruiz et al. 2014). The same applies to the fiber sources that are associated with phytochemicals and antioxidants, such as the

Table 3 Selected functional foods and beverages and lipids in type 2 diabetes: clinical studies

Author, year	Study design	Subject characteristics	Dose and duration	Effects on blood lipids	Effects on blood HbA1c, glucose, insulin, and IR
Rock et al. (2008)	Pre- and post intervention	Patients with T2D ($n = 30$)	WPJ (50 mL/day for 4 weeks) or WPOMxl (5 mL/day for 6 weeks)	Increases in HDL-associated PON1 arylesterase, paraoxonase, and lactonase activities in both groups	No effects
Kar et al. (2009)	RCT	Patients with T2D ($n = 32$)	GSE (600 mg/day) or placebo for 4 weeks	Decreases in total cholesterol	No effects on HOMA-IR; decreases in fructosamine
Mellor et al. (2010)	RCT, crossover	Patients with T2D ($n = 12$)	High or low polyphenol chocolate (45 g/day) for 8 weeks	Decreases in total cholesterol: HDL-C; increase in HDL-C	No effects
Curtis et al. (2012)	RCT	Postmenopausal women with T2D ($n = 93$)	Flavonoid-enriched chocolate (27 g/day) + isoflavones (100 mg/day) or placebo for 1 year	Decreases in total cholesterol: HDL-C and LDL-C	Decrease in HOMA-IR and increased insulin sensitivity
Basu et al. (2013)	Pre- and post intervention	Patients with T2D ($n = 8$)	Pomegranate extracts (~1,400 mg/day) for 4 weeks	No effects	No effects
Liu et al. (2014)	RCT	Patients with T2D ($n = 92$)	500 mg GTE or placebo three times/day for 16 weeks	Decreasing trend in TG only	Decrease in HOMA-IR within group only
Mozaffari-Khosravi et al. (2014)	RCT	Patients with T2D ($n = 100$)	Sour tea or green tea (150 mL three times/day) for 4 weeks	Increase in HDL-C in both groups	Decrease in HOMA-IR and insulin in green tea group
Keith et al. (2015)	Pre- and post intervention	Patients with T2D ($n = 30$)	MPD (viscous fiber, soy protein, almonds) or no diet therapy for 4 weeks	Decrease in LDL-C; no change in HDL-C	No effects

The above table is a summary of intervention studies examining the effects of selected functional foods and beverages on blood glucose, conventional lipids, and lipoprotein subclasses in adults with type 2 diabetes

avenanthramides in oats (Clemen and Klinken 2014). Fruit polyphenols have also been shown to improve dyslipidemia by inhibiting the fat digestion enzymes, though mostly in experimental studies (McDougall and Stewart 2005). While the results from clinical trials hold promise, these lipid-lowering effects of functional foods need further studies in controlled clinical trials of patients with type 2 diabetes. While such research remains underway, it will be prudent to recommend freshly prepared tea, especially green tea, cocoa beverages, and whole fruits, such as berries and pomegranates, and soy protein in the dietary management of diabetic dyslipidemia.

Botanical Extracts and Blood Lipid Control in Diabetes

A large proportion of adults with diabetes use some form of complementary and alternative medicine (CAM) therapy concurrently with conventional health-care measures (Fabian et al. 2011). Spices, such as cinnamon, and traditional herbal extracts are among the popular CAM therapies in diabetes. As summarized in Tables 4 and 5, various botanical extracts, such as cinnamon, garlic, and ginger, and herbal extracts originating from traditional medicinal practices such as Ayurvedic medicine have been shown to lower LDL-C and triglycerides and/or increase HDL-C in most studies of type 2 diabetic patients. Many other traditional herbal supplements, such as the red yeast rice (RYR), a Chinese herbal supplement produced by fermenting white rice with the yeast, have been shown to be safe, effective, and well tolerated; however, the studies are small and of short duration, a major limitation in evidence-based practice related to herbal therapy for diabetes. RYR has been used as an alternative to statin therapy in treating patients with mild to moderate hypercholesterolemia. RYR contains a variety of monacolins, which inhibit hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting step in cholesterol synthesis (Burke 2015). Cinnamon has also been shown to inhibit hepatic HMG-CoA reductase activity and subsequently lower blood lipids in animals and in some human studies (Mang et al. 2006). However, while these findings from human studies show benefits of selected botanical extracts on blood lipid control in diabetic patients, overall evidence on the efficacy and safety of these extracts is insufficient. Further trials, which address study design issues, such as allocation concealment and blinding, interactions of herbs with medication usage, and safety parameters, are needed. The inclusion of other important end points, such as health-related quality of life, diabetes complications, and costs, is also needed to support the use of herbal supplements in the blood lipid management of diabetes.

Potential Applications to Prognosis, Other Diseases, or Conditions

Based on the evidence from clinical and population-based studies, blood lipids and lipoproteins, especially LDL-C, TG, and HDL-C as measures of conventional lipid profiles, as well as detailed NMR-derived lipoprotein subclasses, and modified lipids

Table 4 Cinnamon and lipids in diabetes: clinical studies

Author, year	Study design	Subject characteristics	Cinnamon dose and duration	Effects on blood lipids	Effects on blood HbA1c, glucose, insulin, and IR
Khan et al. (2003)	RCT	Patients with T2D ($n = 60$)	1, 3, or 6 g for 40 days	Decrease in total and LDL-C and TG; no effects on HDL-C	Decreases in serum glucose
Vafa et al. (2012)	RCT	Patients with T2D ($n = 44$)	3 g for 8 weeks	Decrease in TG only	No effects
Allen et al. (2013)	Meta-analysis of ten RCTs	Patients with T2D ($n = 543$)	120 mg/day–6 g/day for 4–18 weeks	Decreases in total and LDL-C and TG; increase in HDL-C	Decreases in plasma glucose; no effects on HbA1c
Azimi et al. (2014)	RCT	Patients with T2D ($n = 204$)	3 g for 8 weeks	Decreases in total and LDL-C; increase in HDL-C	No effects
Beejmohun et al. (2014)	RCT (postprandial)	Healthy volunteers ($n = 18$)	1 g followed by meal and blood draw at 2 h	No effects	Decreases in 2 h postprandial glucose
Whitfield et al. (2015)	RCT	Patients with T2D ($n = 12$)	Honey supplemented with cinnamon, chromium, and magnesium: 53.5 g/day for 40 days	Decreases in total and LDL-C; increasing trends in HDL-C	No effects

The above table is a summary of intervention studies examining the effects of cinnamon extracts on blood glucose, conventional lipids, and lipoprotein subclasses in adults with type 2 diabetes

Table 5 Botanical extracts and lipids in type 2 diabetes: clinical studies

Author, year	Study design	Subject characteristics	Extract dose and duration	Effects on blood lipids	Effects on blood HbA1c, glucose, insulin, and IR
Sobenin et al. (2008)	RCT	Patients with T2D ($n = 60$)	Allicor (time-released garlic powder; 300 mg) or placebo for 4 weeks	Decreases in TG only	Decreases in fasting glucose and fructosamine
Huseini et al. (2012)	RCT	Patients with T2D ($n = 60$)	Aloe gel extracts (300 mg every 12 h) or placebo for 8 weeks	Decreases in total and LDL-C	Decreases in fasting glucose and HbA1c
Kumar et al. (2013)	RCT	Patients with T2D ($n = 60$)	Garlic extracts (250 mg) + metformin or metformin only for 12 weeks	Decreases in total and LDL-C and TG; increase in HDL-C	Decreases in fasting and postprandial blood glucose
Arablou et al. (2014)	RCT	Patients with T2D ($n = 70$)	Ginger extracts (1,600 mg/day) or placebo for 12 weeks	Decreases in total cholesterol and TG	Decreases in fasting glucose, insulin, and HOMA-IR
Yadav et al. (2014)	Pre- and post-intervention trial	Patients with T2D ($n = 58$)	Polyherbal capsules (Diabegon) (4 g/day) or placebo for 18 months	Decreases in total and LDL-C and TG; increase in HDL-C	Decreases in fasting and postprandial glucose
Awasthi et al. (2015)	RCT	Patients with T2D ($n = 93$)	Polyherbal capsules (500 mg/day) or placebo for 24 weeks	Decreases in total cholesterol	Decreases in fasting glucose and HbA1c

The above table is a summary of intervention studies examining the effects of botanical extracts on blood glucose, conventional lipids, and lipoprotein subclasses in adults with type 2 diabetes

such as oxidized LDL play an important role in defining the long-term prognosis of diabetes and atherosclerotic CVD. Clinical trials have demonstrated that intensive lowering of LDL-C is associated with a significant risk reduction of a first major cardiovascular event, defined as death from CHD, nonfatal non-procedure-related

myocardial infarction, resuscitation after cardiac arrest, or fatal or nonfatal stroke (LaRosa et al. 2005; Cannon et al. 2004). Observational studies also suggest that the oxidative modification of LDL may be a marker of metabolic changes preceding or accompanying the onset of T2D (Njajou et al. 2009). Liver dysfunction of lipoprotein metabolism is mechanistically associated with diabetes (Sonmez et al. 2015), and thus clinical studies must further address the role of phytochemicals in reversing the mechanisms by which excess hepatic lipid develops and causes hepatic insulin resistance and type 2 diabetes. Proposed mechanisms implicate various lipid species, inflammatory signaling, and other cellular modifications in diabetes (Njajou et al. 2009). Oxidized LDL has also been positively associated with obesity and inflammation, and among individuals with elevated abdominal fat, the effect of oxidative stress was even greater on CVD risk (Sonmez et al. 2015; Weinbrenner et al. 2006). Thus, keeping in view the value of lipid and lipoprotein biomarkers in the prognosis of diabetic vascular complications, the role of plant-based diets and phytochemical-containing foods and beverages in improving atherogenic lipid profiles deserves special attention in nutrition interventions for diabetes. Future studies must address the role of these phytochemical-based foods and diets in modulating lipid profiles at various stages of diabetes complications and their associations with other novel biomarkers, such as those related to lipidomics, proteomics, and metabolomics, in the prognosis and management of vascular complications in diabetes.

Summary Points

- Biomarkers of blood lipids and lipoproteins play an important role in prognosis and management of vascular complications of diabetes.
- Diabetic dyslipidemia is characterized by quantitative and qualitative changes in lipids, such as high levels of LDL-C and triglycerides and low HDL-C, and oxidation of LDL.
- Plant-based diets can effectively lower LDL-C and triglycerides and increase HDL-C in type 2 diabetes.
- Phytochemical-rich functional foods and beverages such as fruits, soy, and tea can lower LDL-C and increase the protective functions of HDL against vascular complications of diabetes.
- Specific functional foods and plant compounds, such as soy, nuts, and oats, have health claims by FDA based on substantial evidence on their cholesterol-lowering effects in clinical studies.
- Herbs and spices such as cinnamon and garlic have been shown to lower lipids and glucose in some studies but need further research for their recommendation in diabetic dyslipidemia.

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Gamma Glutamyltransferase (GGT) as a Biomarker of Atherosclerosis

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_50

Abstract

Gamma-glutamyltransferase (GGT) is an ubiquitous enzyme in human tissues that recycles precursors to the antioxidant and metabolic substrate, glutathione (GSH). GSH is critical in the dynamic preservation of antioxidant balance and in the elimination of xenobiotic substrates. When the total GSH pool becomes limited, either due to increased exposure to oxidative challenges or demand for detoxification, GGT activity increases. Related to atherosclerosis, GSH demand increases with exposure to dietary and environment exposures that have been implicated in vascular inflammation and subsequent atherosclerosis including dietary iron, lipid peroxides, advanced glycation end (AGE) products, and reduced antioxidant intake. Additional stressors on GSH balance include environmental contaminants such as persistent organic pollutants and heavy metals, which also contribute to vascular inflammation directly and indirectly due to metabolic disruption. The results of examinations of several cross-section and longitudinal cohort studies, including our results in the Multi-Ethnic Study of Atherosclerosis (MESA), demonstrate strong associations with: individual risk factors, composite cardiometabolic conditions, mechanistic atherosclerotic biomarkers, and cardiovascular events. Evaluated in totality, the existing evidence strongly suggests GGT activity is a biomarker of systemic oxidative demand indicative of active vascular inflammation, metabolic compromise, and atherosclerosis.

Keywords

Oxidative stress • Inflammation • Oxidized LDL • Endothelial dysfunction • Atherosclerosis • Adhesion molecules • IL-6 • sICAM-1 • CRP

Abbreviations

4-HNE	4-Hydroxynonal
AGE	Advanced glycation end products
CARDIA	Coronary Artery Risk Development in Young Adults CARDIA
CRP	C-Reactive protein
FBG	Fasting blood glucose
FI	Fasting insulin
FMD	Flow-mediated dilation
GGT	Total serum γ-glutamyltransferase activity
GSH	Glutathione
HbA1c	Hemoglobin A1c
HOMA-IR	Homeostasis assessment index
IL-6	Interleukin 6
MESA	Multi-Ethnic Study of Atherosclerosis
NAC	n-Acetylcysteine
oxLDL	Oxidized LDL
sICAM-1	Soluble intercellular adhesion molecule

Key Facts of GGT in Relation to Atherosclerosis and Cardiovascular Disease

- GGT appears to represent total body demand for a key antioxidant molecule called glutathione.
- Studies in large groups of adults performed across the world in many ethnic groups suggest GGT activity is related to increased risk for cardiovascular disease, including male gender, higher risk ethnic subgroups, age, smoking history, waist circumference, LDL, triglycerides, blood pressure, and use of medications for diabetes, hypertension, and cholesterol.
- GGT is associated with multiple indicators of atherosclerosis, including oxidized LDLs, markers of inflammation, markers of immune system contribution to plaque development, and binding molecules that indicate atherosclerosis.
- GGT activity is also associated with conditions that are a significant risk for developing cardiovascular disease, specifically type 2 diabetes and the metabolic syndrome.
- Individual risk factors for developing diabetes are also associated with GGT, including blood glucose (FBG); insulin; average blood sugar, measured by hemoglobin A1c (HbA1c); and models of insulin resistance (HOMA-IR).
- Significant trends for increased prevalent metabolic disease were evident in all ethnic groups (i.e., White, Black, and Hispanic), except Chinese.
- Age greater than 65 years reduced the strength of the associations between GGT activity and disease risk.

Definitions

Advanced glycation end (AGE) products Chemical modification of food that forms from the nonenzymatic reaction between carbohydrates and proteins during cooking at high temperatures in the presence of oxygen.

C-reactive protein (CRP) An acute phase reactant associated with immune stimulation and increased risk of cardiovascular events.

Glutathione An antioxidant peptide consisting of glycine, cysteine, and glutamate that serves as a substrate for the enzyme glutathione peroxidase (GPx) in the reduction of lipid peroxides, and the enzyme glutathione-s-transferase (GST), which conjugates glutathione to xenobiotic compounds for elimination.

HOMA-IR Homeostasis assessment index of insulin resistance, a mathematical model to estimate in vivo insulin resistance based on fasting glucose and fasting insulin or c-peptide.

Interleukin-6 (IL-6) Immune cytokine that triggers acute phase inflammation and the release of acute phase reactants in the liver.

Lipid peroxides Chemical modification of fatty acids, especially unsaturated fatty acids, when cooked at high temperatures. Lipid peroxides are substrates of glutathione-s-transferase, and thus require glutathione for elimination.

***N*-acetylcysteine (NAC)** An antioxidant that provides cysteine for the production of glutathione.

Oxidized LDL (oxLDL) Oxidized low density lipoproteins are chemically oxidized lipoprotein particles that bind receptors and contribute to the formation of atherosclerotic plaques.

Soluble intracellular adhesion molecules (sICAM-1) Endothelial receptors that bind white blood cells during the process of atherosclerosis.

γ–Glutamyltransferase (GGT) An enzyme, previously considered a liver enzyme, that helps recycle precursors to the antioxidant peptide, glutathione.

Introduction

There has been a resurgence of interest in the enzyme γ-glutamyltransferase (GGT) due to the results of several observational studies identifying associations between graded elevations in its activity in serum and increased risk of adverse cardiovascular and metabolic outcomes, including metabolic syndrome (Liu et al. 2012b), type 2 diabetes (Andre et al. 2006; Lim et al. 2007; Meisinger et al. 2005; Nguyen et al. 2011; Onat et al.; Fraser et al. 2009), hypertension (Onat et al.; Liu et al. 2012a), congestive heart failure (Wannamethee et al.), and vascular events (Meisinger et al. 2006; Fraser et al. 2007), plus increased mortality from cardiovascular disease and diabetes (Ruhl and Everhart 2009; Lee et al. 2009). The known physiologic function of GGT is to contribute to in vivo antioxidant homeostasis through recycling extracellular glutathione (GSH), and its precursor amino acids, for intracellular reconversion to reduced GSH (Dickinson and Forman 2002). The tripeptide reduced glutathione (GSH) is a critical antioxidant defense in human tissues; in the absence of adequate GSH, elevations in superoxide, peroxide, and peroxynitrite free radicals persist causing lipid peroxidation, protein modification, and DNA adduct formation with varying consequences on membrane receptor and gene functioning, including impaired endothelial-mediated vasodilation (Franco et al. 2007). Vascular inflammation and oxidative stress have been implicated in the origins of endothelial dysfunction, which contributes to the microvascular complications of metabolic disease and atherosclerotic disease of the macro-vasculature (Evans et al. 2002; Zambon et al. 2005; Touyz 2005; De Mattia et al. 2008). Endothelial dysfunction is a cumulative process secondary to increased concentrations of, and variability in, blood glucose and lipids, with subsequent redox dysregulation (Ceriello 2000; Ceriello et al. 2002). Relevant biomarkers of oxidation, immune activation, and

subclinical inflammation include malondialdehyde modified low-density lipoproteins (commonly referred to as "oxidized" LDL or oxLDL), cytokines such as interleukin-6 (IL-6), elevations in acute phase inflammatory biomarkers including C-reactive protein (CRP), and increased soluble vascular adhesion molecule (sICAM-1) expression- biomarkers which have all demonstrated increased risk prediction beyond traditionally established risk factors (Pereira et al. 2008). However, biomarkers of oxidative-inflammatory stress have limitations in clinical research due to the need for careful sample handling, instrumentation requirements, and the high costs of measurement- factors which limit investigation of these processes in population-based studies of human disease, and create barriers to translating basic science evidence into clinical research (Mayne 2003). GGT provides: a rapid, inexpensive, clinically available biomarker to assess physiologic demand for antioxidant substrates; an indicator of composite dietary and environmentally associated disease risk; and direct insight into the risk for adverse cardiovascular and metabolic disease outcomes in at-risk populations.

The Physiologic Action of γ–Glutamyltransferase (GGT)

The primary action of GGT in vivo is to facilitating transmembrane transport of cysteine and other precursors of the antioxidant peptide glutathione for reconversion into intracellular glutathione (GSH) (Dickinson and Forman 2002). Intracellular glutathione is a critical antioxidant defense; in the absence of adequate glutathione lipid peroxidation, protein modification and DNA adduct formation occur with varying consequences on membrane receptor and gene function. Intrahepatic glutathione also serves as an important conjugation substrate for the elimination of xenobiotics. The specific relationship between GGT and in vivo redox balance has been extensively reported by Whitfield (2001), is summarized in our Multi-Ethnic Study of Atherosclerosis (MESA) findings (Bradley et al. 2013).

The Validity of GGT Measurement

There is no "gold-standard" biomarker for systemic oxidative stress in human populations. The evidence available on GSH status and cardiovascular risk is limited to patients with type 2 diabetes, and demonstrates lower concentrations of GSH (measured as erythrocyte GSH) in those with diabetes compared to those without diabetes, and diabetics with microvascular complications appear to have still lower concentrations, suggesting the importance of GSH status in complication development (De Mattia et al. 2008; Ahmadpoor et al. 2009; Thornalley et al. 1996). Although these relatively small case–control studies have demonstrated differences in GSH status, the validity of erythrocyte GSH as a biomarker remains mostly unknown in large population-based studies of cardiovascular disease. Unfortunately, the need to preserve reduced GSH in stored specimens at the time of collection impedes post-hoc measurement of erythrocyte GSH in stored

samples, and thus limits evaluations of GSH in cardiovascular disease cohorts (Tietze 1969).

Alternatively, the case for the validity of GGT as a clinically significant biomarker of oxidative stress has gained strength. In order for a biomarker of systemic (i.e., multiple tissues) oxidative stress to be valid in metabolic disease one would expect the biomarker to: (1) Have supporting mechanistic data that supports the role of the biomarker in relation to oxidative stress development, (2) Correlate to existing gold-standard biomarkers of biomolecule oxidation (e.g., F2-isoprostanes) for lipid peroxidation, (3) Correlate with known risk factors for metabolic disease including lifestyle factors, (4) Increase with increasing risk for metabolic disease, and (5) Continue to correlate with oxidation-linked complications in relevant disease states.

Several available research results provide a convincing rationale that increased GGT activity represents increased "oxidative stress" via increased demand for GSH. Findings supportive of this role include: (1) GSH depletion appears to be a prerequisite condition to induce GGT (Braide 1989); (2) NADPH oxidase-produced ROS, and reactive nitrogen species both induce GGT expression (Huseby et al. 2003; Ravuri et al. 2011); (3) mitochondria of GGT-knock-out mice have depleted GSH, increased reactive oxygen species (ROS) formation, depleted energy stores, and impaired oxidative phosphorylation (thus impaired ATP production), which can be attenuated by *N*-acetylcysteine (NAC), a GSH precursor (Will et al. 2000); and (4) GGT-knock-out mice die prematurely with complications associated with increased oxidative stress- similar to complications in type 2 diabetes (e.g., cataracts and microvascular compromise) (Chevez-Barrios et al. 2000). Additional animal in vivo data support the relationship between GGT activity and oxidative stress induction. Watkins et al. reported increased GGT activity in streptozotocin (STZ)-induced diabetic rats (Watkins et al. 1998). STZ is a potent alkylating agent that induces diabetes in the rat in part through depletion of pancreatic glutathione concentration. In addition, GGT knock-out mice have reduced glutathione levels compared to controls, develop cataracts and die prematurely unless treated with glutathione precursors (Chevez-Barrios et al. 2000). These data provide mechanistic support for the role of GGT in offsetting damage caused by oxidative stress. Translating this hypothesis to research in humans, and further supporting a relationship between GGT and GSH, Sedda et al. demonstrated inverse associations between GGT activity and plasma total GSH concentration in people with established cardiovascular risk, and after multivariate adjustment for individual risk factors, plasma total GSH remained the only independent variables associated with GGT activity (Sedda et al. 2008).

Regarding the relationship between GGT and "gold-standard" markers of oxidative stress in humans, lipid peroxidation- both endogenous and exogenous-contributes to glutathione demand. GSH is required for the elimination of lipid peroxides, and thus increases requirements for GSH. Peroxidation is thought to contribute to vascular injury and endothelial dysfunction. Positive associations were found between biomarkers of lipid peroxidation and GGT in the Coronary Artery Risk Development in Young Adults (CARDIA) cohort (Lee et al. 2003). Specifically, increasing percentiles of GGT (although still within the normal

range) were associated with F2-isoprostanes, in both men and women after adjustment for study center, race, age, and sex. In addition to positive correlations with isoprostanes, positive associations were also found with C-reactive protein, a recognized measure of systemic inflammation. Additional support for the validity of GGT as a marker for systemic oxidative stress in humans comes from the Tromso cohort study, which measured GGT in over 21,000 men and women and evaluated population determinants of GGT activity (Nilssen et al. 1990). Positive associations were found between GGT activity and alcohol, body mass index, and total cholesterol. Inverse associations were found between GGT activity and increased physical activity and the consumption of coffee; as mentioned coffee is known to increase erythrocyte glutathione levels (Esposito et al. 2003).

Laboratory Measurement of GGT

GGT (as activity) is measured in human plasma or serum. Measurement of GGT has been used clinically for many years as a sensitive indicator of hepatobiliary disease, including alcoholism and bile duct obstruction. Because of its role in clinical diagnostic testing, Clinical Laboratory Improvement Amendments (CLIA) and International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) testing standards are in place and CLIA/IFCC-certified automated, multitest instruments that test GGT are readily available in clinical laboratories and exhibit good quality control. Published day-to-day coefficients of variability for example multitest instruments are <5 % (Forouhi et al. 2007; Steinmetz et al. 2007).

The assay used to measure GGT is technically a measure of GGT activity, not concentration or expression; tissue GGT expression has been measured using northern and western blot (Watkins et al. 1998). Figure 1 outlines the chemical reaction used to measure GGT activity. In brief, an amino acid residue is transferred by GGT from gamma-glutamyl-p-nitroanilide to a small peptide, producing p-nitroaniline, which is measured using a visible spectrum photometer at 405 nm. The resulting absorbance is then compared to known GGT standards for report of GGT activity (Lee et al. 2003).

The Reliability of GGT Measurement

One of the earliest reported reliability studies on GGT was performed by Rhone and White in 1976 (Rhone and White 1976). In their study, they collected blood from 10 participants and split the samples, storing half of the sample at 4 °C with GGT

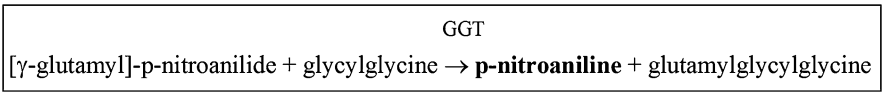

Fig. 1 Chemical reaction used to measure GGT in blood samples

measurement at 1, 2, 5, and 9 weeks. The other aliquot they stored at −6 °C with thawing and GGT measurement at 1, 2, 6, 10, and 40 weeks. Although the authors did not calculate reliability coefficients, they report that of the 50 GGT measurements from samples stored at 4 °C, only 15 showed a 10 % or greater change in GGT activity. For those samples stored at −6 °C, no statistically different changes in GGT activity were measured using paired analyses, however a reliability coefficient was not calculated. Although the data presented by Rhone and White suggest low variability in GGT activity with proper samples storage (freezing), their data is not completely interpretable due to the lack of reporting of reliability coefficients.

Better data regarding the reliability of GGT is available from the CARDIA cohort (Lee et al. 2003). CARDIA followed 5,115 adults between 18 and 30 years of age for 15 years. GGT was measured at year 0 and year 10. Because of differences in instrumentation between year 0 and year 10, the investigators remeasured GGT in 103 year 0 samples. This remeasurement happened after 17 years of sample storage at −70 °C. They reported a reliability coefficient of 0.995 between year 0 measured samples and the 103 remeasured year 0 samples. This high reliability coefficient supports high intra-sample and inter-method reliability for GGT. In addition it suggests the effects of storage were negligible. The number of freeze-thaw cycles for the samples in the interim period, i.e., year 0 to year 17, was unstated. In addition, the investigators calculated a reliability coefficient of 0.67 between year 0 samples and year 10 samples, suggesting good inter-sample reliability.

GGT as a "Liver Enzyme"

Commonly classified as a "liver enzyme" associated with gall bladder disease, alcoholism, and frank hepatitis, liver tissue GGT expression does not always correlate with *serum* GGT activity (Satoh et al. 1980; Selinger et al. 1982). GGT is expressed in multiple human tissues other than the liver, with total serum GGT activity corresponding to the sum of activity from at least four fractions (Franzini et al. 2008, 2009, 2012, 2013a; Paolicchi et al. 2006). Methods for measuring subfractions of total GGT activity (i.e., *s-GGT, m-GGT, b-GGT, and f-GGT* for "small," "medium," "big" and "free," respectively) have been reported by Franzini et al. (2008), and disease-specific patterns of GGT subfractions and ratios of subtypes are emerging, e.g., increased *b-GGT* has been recently associated with cardiovascular risk (Franzini et al. 2013a), whereas *s-GGT* was increased in alcoholics (Franzini et al. 2013b). Subfractionation should be applied in future studies to better differentiate subgroups within larger cohorts by disease status, including those with isolated hepatic disease and those with multiorgan disease, i.e., concomitant nonalcoholic fatty liver disease, metabolic disease, *and* cardiovascular disease. The innovative method of GGT subfractionation will also be critical in future determinations of the organ-specific mechanisms responsible for observed increases in serum GGT activity. Despite the potential to improve upon our methods by subfractionating GGT, the associations demonstrated here with total serum GGT

activity remain valid. Other studies have demonstrated that participants with increased *total* serum GGT activity have increased GGT subfractions more associated with disease (i.e., non-*f-GGT fractions*); total serum GGT activity increased with increases in each subfraction (i.e., except *f-GGT)*; and total serum GGT activity remains the most accessible biomarker to clinicians (Franzini et al. 2012, 2013a, b). However, despite the insights gained from fractionating GGT, the relationship between *tissue* GGT activity and *serum* GGT activity remains under active investigation.

GGT Activity and Atherosclerosis

GGT Activity and Individual Risk Factors for Cardiovascular Disease

Our investigations in the Multi-Ethnic Study of Atherosclerosis (MESA) began with detailed evaluations of potential cross-sectional associations between GGT and established risk factors for atherosclerosis and cardiovascular disease, including demographics, lifestyle variables, and clinical/laboratory risk factors (Bradley et al. 2014). The results are consistent, demonstrating GGT is strongly and positively associated with nearly all established risk factors (Table 1), including male gender, at-risk ethnic subgroups, smoking status, increased alcohol intake, increased LDL, reduced HDL, elevated triglycerides, increased insulin resistance, and both increased systolic and diastolic blood pressures. The consistency of these findings suggests a common mechanism underlying these associations, such as vascular inflammation secondary to oxidative stress, immune activation, and inflammation.

GGT Activity and Increased Risk for Adverse Metabolic and Cardiovascular Outcomes

The results of numerous observational studies, e.g., NHANES III, CARDIA, EPI-Norfolk, have identified associations between graded elevations in its activity in serum and increased risk of adverse cardiovascular and metabolic outcomes, including metabolic syndrome (Liu et al. 2012b; Bradley et al. 2013), type 2 diabetes (Andre et al. 2006; Lim et al. 2007; Meisinger et al. 2005; Nguyen et al. 2011; Onat et al.; Fraser et al. 2009; Bradley et al. 2013), hypertension (Onat et al.; Liu et al. 2012a), congestive heart failure (Wannamethee et al.), and vascular events (Meisinger et al. 2006; Fraser et al. 2007), plus increased mortality from cardiovascular disease and diabetes (Ruhl and Everhart 2009; Lee et al. 2009).

In order to connect GGT activity specifically with increased vascular risk, our work tested a mechanistic conceptual model in the MESA cohort hypothesizing GGT activity would be independently associated with biomarkers of oxidation, immune activation, and atherosclerosis (Bradley et al. 2014). We measured significant, positive associations between GGT activity and increased oxidized LDL (oxLDL), C-reactive protein (CRP), interleukin-6 (IL-6), and intravascular adhesion molecule-1 (ICAM-1);

Table 1 Associations between GGT, demographic variables, and cardiometabolic risk factors

Baseline characteristic (n = 6,446)	GGT-Q1 <24.5 U/l *n* (%) or Mean (SD)	GGT-Q2 24.5–29.3 U/l *n* (%) or Mean (SD)	GGT-Q3 29.3–35.1 U/l *n* (%) or Mean (SD)	GGT-Q4 35.1–45.2 U/l *n* (%) or Mean (SD)	GGT-Q5 >45.2–99.7 U/l *n* (%) or Mean (SD)	*P*-value for trend
Age (year)	62.4 (10.9)	63.5 (10.3)	62.8 (10.0)	62.1 (10.0)	61.0 (9.7)	<0.0001
Gender: Male	325 (25.2 %)	523 (40.7 %)	635 (49.1 %)	724 (52.5 %)	840 (65.1 %)	<0.0001
Race White Chinese Black Hispanic	665 (30.0 %) 194 (21.9 %) 226 (12.9 %) 204 (14.5 %)	543 (24.5 %) 173 (19.6 %) 308 (17.6 %) 262 (18.6 %)	465 (20.9 %) 164 (18.6 %) 389 (22.3 %) 274 (19.5 %)	435 (19.6 %) 123 (13.9 %) 430 (24.6 %) 301 (21.4 %)	408 (18.4 %) 119 (13.5 %) 395 (22.6 %) 368 (26.2 %)	Referent 0.91 <0.0001 <0.0001
Smoking status Never Current Past	746 (22.9 %) 105 (14.9 %) 432 (18.2 %)	663 (20.4 %) 135 (19.2 %) 483 (20.4 %)	695 (21.4 %) 159 (22.6 %) 435 (18.3 %)	615 (18.9 %) 172 (24.4 %) 498 (21.0 %)	533 (16.4 %) 228 (32.4 %) 525 (22.1 %)	Referent <0.0001 <0.0001
Pack years (year)	21.1 (23.2)	21.6 (21.6)	24.8 (27.1)	23.4 (25.5)	24.5 (33.7)	0.021
Current alcohol use (%)	699 (10.3 %)	669 (9.8 %)	672 (10 %)	719 (10.6 %)	782 (11.5 %)	0.34
Current drinks/week	2.4 (3.7)	3.5 (5.1)	3.5 (5.1)	4.4 (6.8)	5.9 (8.0)	<0.0001
Physical activity (total MET-min/Week)	1,499 (2,131)	1,529 (2,286)	1,548 (2,196)	1,558 (2,580)	1,609 (2,841)	0.75

Diabetes medications None Insulin Oral	1,218 (94 %) 11 (1 %) 60 (5 %)	1,184 (92 %) 19 (2 %) 83 (6 %)	1,159 (89 %) 20 (2 %) 113 (9 %)	1,150 (89 %) 21 (2 %) 118 (9 %)	1,124 (87 %) 33 (3 %) 133 (10 %)	Referent <0.0001 <0.0001
Total cholesterol (mg/dl)	192.0 (32.6)	192.8 (35.7)	193.5 (35.3)	195.3 (34.8)	196.8 (37.6)	<0.0001
LDL-C (mg/dl)	114.1 (29.4)	116.6 (31.1)	118.3 (31.0)	119.6 (31.9)	118.7 (32.5)	<0.0001
HDL-C (mg/dl)	56.7 (15.3)	52.4 (15.5)	49.4 (14.1)	48.4 (13.3)	48.0 (13.8)	<0.0001
Triglycerides (mg/dl)*	94.0 (68–127)	104.0 (74–146)	111.0 (78–158)	119.0 (83–174)	132.0 (89–191)	<0.0001
Lipid-lowering medications	181 (14 %)	203 (16 %)	213 (16.5 %)	230 (18 %)	222 (17 %)	0.036
SBP (mm Hg)	121.9 (21.6)	125.7 (22.0)	127.7 (21.7)	128.1 (21.0)	129.2 (20.5)	<0.0001
DBP (mm Hg)	68.3 (10.0)	70.4 (9.9)	72.4 (10.1)	74.4 (10.1)	73.9 (10.2)	<0.0001
Current HTN	466 (36 %)	531 (41 %)	591 (46 %)	642 (50 %)	650 (50.4 %)	<0.0001
HTN medications	395 (31 %)	454(35 %)	485 (38 %)	537 (42 %)	517(40 %)	<0.0001
Waist circumference (cm)	91.9 (14.3)	96.2 (14.3)	99.0 (14.0)	101.1 (13.8)	101.4 (13.4)	<0.0001
Glucose (mg/dl)*	85.0 (80–91)	88.0 (82–95)	91.0 (84–100.5)	92.0 (85–103)	93.0 (86–106)	<0.0001
Insulin (μU/l)*	3.8 (2.7–5.5)	4.8 (3.3–7.2)	5.6 (3.8–8.6)	6.2 (4.1–9.4)	7.1 (4.4–11.1)	<0.0001

*denotes a skewed variable; descriptive statistics reported as median and inter-quartile range (IQR).
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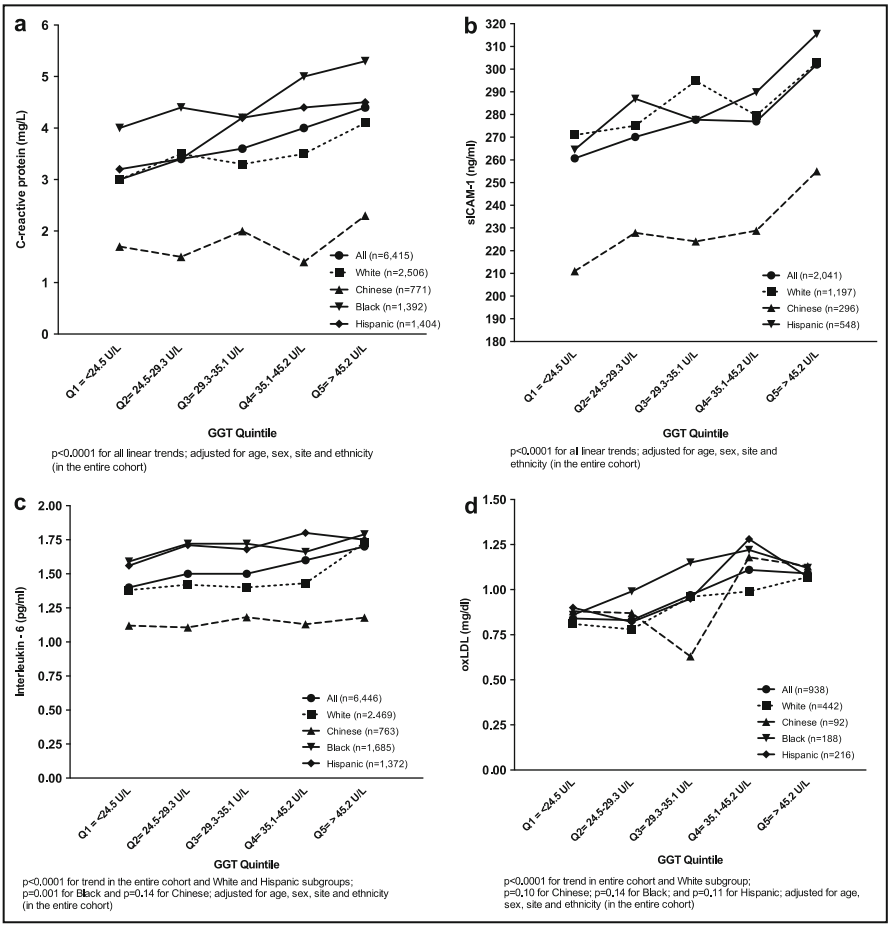

Fig. 2 Associations between GGT and biomarkers of atherosclerosis. Associations between GGT activity and biomarkers of atherosclerosis including: C-reactive protein (**a**), soluble intravascular adhesion molecules (**b**), interleukin-6 (**c**), and oxidized LDL (**d**) (Reprinted with permission from Elsevier Publishing)

see Fig. 2 and Table 2. The overall relationship identified was consistent across ethnicities, with rare exceptions. Specifically, we saw evidence of several significant associations between γ-glutamyltransferase (GGT), traditional cardiovascular risk factors, and biomarkers of oxidative stress, immune activation, acute phase response, and endothelial dysfunction. Specifically, we saw evidence of strong associations for increasing trends in oxLDL, IL-6, CRP, and sICAM-1 with graded increases in GGT in the entire cohort. Continuous associations between GGT and all biomarkers of interest were significant after adjustment for age, race, gender, and study site, and remained significant for IL-6, CRP, and sICAM-1 after adjustment for risky lifestyle factors and

Table 2 Adjusted associations between GGT and biomarkers of atherosclerosis: CRP, IL-6, oxLDL, and sICAM-1

CRP = $\beta_{Adj.}$*GGT + $\beta_n X_n \ldots + \beta_0$, n = 6,415	$\beta_{Adj.}$[a]	95 % CI	p-value for GGT
M1 (n = 6,415)	0.042	0.03–0.05	<0.0001
M2 (n = 3,819)	0.032	0.02–0.04	<0.0001
M3 (n = 3,774)	0.025	0.01–0.04	<0.0001
M4 (n = 3,772)	0.017	0.005–0.03	0.006
IL-6 = $\beta_{Adj.}$*GGT + $\beta_n X_n \ldots + \beta_0$, n = 6,289	$\beta_{Adj.}$	95 % CI	p-value for GGT
M1 (n = 6,289)	0.0069	0.005–0.009	<0.0001
M2 (n = 3,745)	0.0065	0.004–0.009	<0.0001
M3 (n = 3,701)	0.0060	0.003–0.009	<0.0001
M4 (n = 3,699)	0.0036	0.001–0.006	0.007
oxLDL = $\beta_{Adj.}$*GGT + $\beta_n X_n \ldots + \beta_0$, n = 935	$\beta_{Adj.}$	95 % CI	p-value for GGT
M1 (n = 935)	0.005	0.002–0.008	0.004
M2 (n = 583)	0.006	0.003–0.010	0.001
M3 (n = 573)	0.001	−0.001–0.004	0.41
M4 (n = 572)	0.0007	−0.002–0.004	0.64
sICAM-1 = $\beta_{Adj.}$*GGT + $\beta_n X_n \ldots + \beta_0$, n = 938	$\beta_{Adj.}$	95 % CI	p-value for GGT
M1 (n = 938)	0.87	0.68–1.06	<0.0001
M2 (n = 585)	0.68	0.45–0.91	<0.0001
M3 (n = 575)	0.68	0.45–0.93	<0.0001
M4 (n = 574)	0.51	0.27–0.75	<0.0001

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M1 adjusts for age (years), gender, ethnicity, and study site

M2 equals M1 + alcohol use (current/former/never and drinks/week) + exercise (MET-min/week) + smoking (current/former/never and pack years)

M3 equals M2 + lipids (LDL-C, HDL-C, triglycerides in mg/dl), lipid-lowering medications, systolic and diastolic blood pressures (mmHg), antihypertensive medications, diabetes medications, and family history of heart attack or stroke

M4 equals M3 + waist circumference (cm), fasting blood glucose (mg/dl), and fasting insulin (μU/l)

[a]Corresponding units for regression coefficients are: CRP: mg/L/unit GGT activity; IL-6: pg/ml/unit GGT activity; oxLDL: mg/dl/unit GGT activity; and sICAM-1: ng/ml/unit GGT activity

traditional risk factors. Although the strength of all associations was attenuated by adjustment for metabolic status, most associations remained significant and independent. Associations between GGT and oxLDL were significant in the entire cohort after adjustment for demographics and risky lifestyle factors, but not following adjustment for traditional risk factors, likely secondary to moderate colinearity between LDL and oxLDL (i.e., correlation coefficient = 0.58 between LDL-C and ln(oxLDL) in MESA). Our findings are strengthened by the use of the MESA cohort for our analyses, which is unique amongst population cohorts in its excellent ethnic representation and

its collection of emerging biomarkers like oxLDL. No known prior studies have evaluated associations between GGT and oxLDL, IL-6, and sICAM-1, nor have any prior studies evaluated associations with CRP in ethnic subgroups.

These findings are supported by those of prior investigations in the CARDIA cohort study which demonstrated similar associations between GGT and CRP and fibrinogen, as well as additional support for GGT associations with lipid peroxidation products, e.g., F2-isoprostanes (Lee et al. 2003). Combined with our findings confirming associations with CRP, and demonstrating novel associations with oxLDL, IL-6, and sICAM-1, these data support the hypothesis that increased GGT activity represents immune stimulation (i.e., IL-6) of hepatic acute phase response (i.e., CRP and fibrinogen), possibly due to increased lipid peroxidation products (i.e., oxLDL and F2-isoprostanes) (Il'yasova et al. 2008; Zhang et al. 2012). Our findings extended this conceptual model to include vascular endothelial involvement, i.e., sICAM-1.

Because nonalcoholic fatty liver disease and other hepatic disease are known to be associated with adverse cardiovascular outcomes, at first glance findings appear limited in that we do not adjust for liver function, i.e., serum AST or ALT, in our analyses. Unfortunately, neither AST nor ALT data are available in the MESA cohort for inclusion. While adjustment for AST and ALT would have assisted in reducing the contribution of hepatic disease to our findings, as suggested in our prior work (Bradley et al. 2013), serum GGT activity is not correlated with hepatic expression of GGT (Selinger et al. 1982). However, we partially accounted for liver disease in two ways: by restricting our analyses to the lower 95th percentile of the GGT activity range (thus likely eliminating severe hepatic disease) and by adjusting for continuous alcohol intake. We were not interested in adjusting our results for fatty liver disease, as fatty liver is highly correlated with hepatic insulin resistance, and thus adjusting for it would have eliminated the influence of metabolic risk fundamental to our hypothesis.

In addition to our research in MESA developing a mechanistic model connecting GGT to atherosclerosis, we also evaluated the relationship between GGT and clinical models of extreme oxidative stress and vascular risk – namely metabolic syndrome and type 2 diabetes (Bradley et al. 2013). GGT activity is strongly associated with individual and composite cardiovascular and metabolic risk factors in the MESA cohort (Fig. 3 and Tables 3 and 4). The observed increases in prevalent metabolic diseases across GGT quintiles are highly consistent with our unadjusted analyses demonstrating positive associations between GGT and "riskier" profile for nearly every individual risk variable (Table 1). The results of our multivariable analysis suggest the associations between GGT and individual metabolic risk factors, especially fasting insulin and HOMA-IR, are very stable and remain independent of standard clinical measures and lifestyle variables. Similar to previous reports, we measured a significant interaction between GGT, BMI, and HOMA-IR, and weaker associations in adults over 65 years of age (Lim et al. 2007; Lee et al. 2009). Because these associations are so consistent, strongly significant, and relatively independent of ethnic group, we suggest total serum GGT activity is a continuous generalizable biomarker of composite metabolic risk in those adults without clinically evident vascular

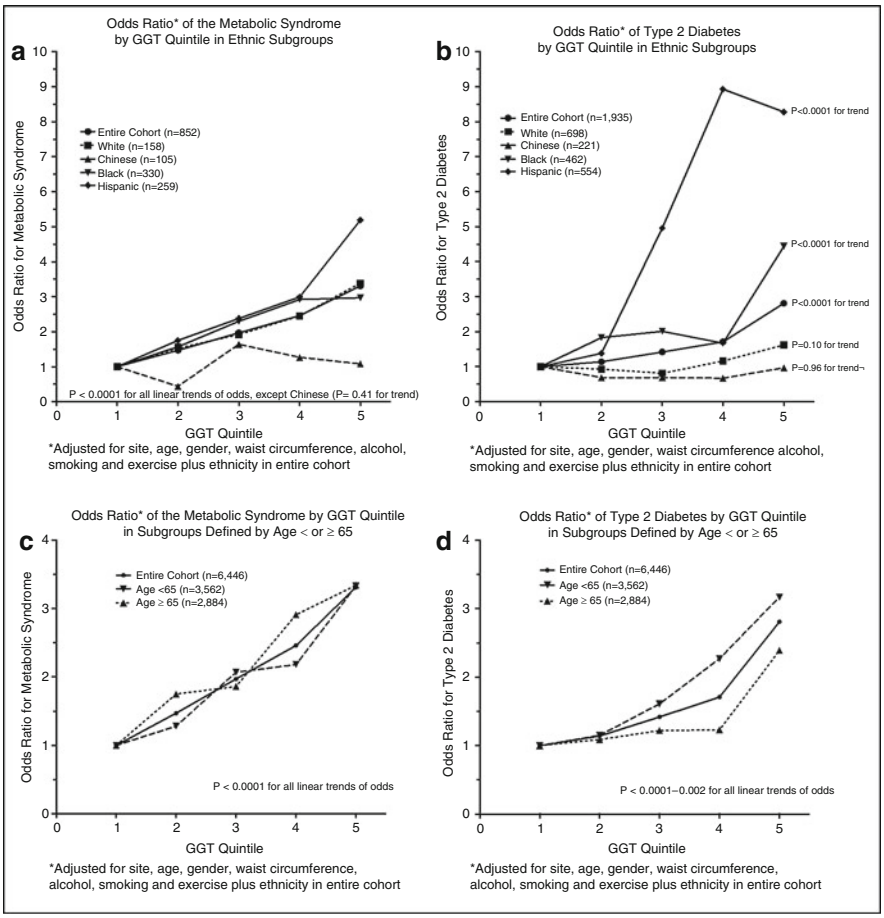

Fig. 3 Odd ratios of metabolic disease by GGT quintile in subgroups defined by ethnicity and age. Demonstration of increased risk of type 2 diabetes and metabolic syndrome by ethnic and age subgroups

disease, with particular utility in people under age 65 years, even after considering differences in established demographic, behavioral, and clinical risk factors.

GGT Activity and Dietary Patterns Associated with Cardiovascular Disease and Diabetes

One explanation regarding sources of increased oxidative stress and endothelial dysfunction in the general population is the diet, and specifically dietary characteristics, including nutritional composition, as well as, preparation methods.

Table 3 Adjusted odds ratios of cardiometabolic disease by GGT quintile in the entire MESA cohort and stratified by ethnic groups

Cardiometabolic subgroup	GGT-Q1 <24.5 U/l Referent group Prevalence	GGT-Q2 24.5–29.3 U/l OR[a] (95 % CI) *P*-value[b] Prevalence	GGT-Q3 29.3–35.1 U/l OR[a] (95 % CI) *P*-value[b] Prevalence	GGT-Q4 35.1–45.2 U/l OR[a] (95 % CI) *P*-value[b] Prevalence	GGT-Q5 45.2–99.7 U/l OR[a] (95 % CI) *P*-value[b] Prevalence	*P*-value for trend	*P*-value for interaction with ethnicity
Metabolic syndrome All ethnicities ($n = 1{,}151$)	Referent $n = 154$	1.47 (1.09–1.99) 0.01 $n = 210$	1.97 (1.47–2.65) <0.0001 $n = 246$	2.46 (1.83–3.30) <0.0001 $n = 256$	3.31 (2.46–4.46) <0.0001 $n = 285$	<0.0001	–
White ($n = 466$)	Referent $n = 76$	1.53 (1.03–2.29) 0.04 $n = 97$	1.92 (1.27–2.91) 0.002 $n = 97$	2.45 (1.63–3.70) <0.0001 $n = 90$	3.39 (2.24–5.14) <0.0001 $n = 106$	<0.0001	Referent
Chinese ($n = 127$)	Referent $n = 22$	0.44 (0.12–1.56) 0.21 $n = 25$	1.64 (0.52–5.17) 0.40 $n = 32$	1.27 (0.39–4.19) 0.69 $n = 26$	1.08 (0.30–3.87) 0.90 $n = 22$	0.41	0.34
Black ($n = 229$)	Referent $n = 24$	1.57 (0.76–3.26) 0.22 $n = 29$	2.30 (1.16–4.56) 0.017 $n = 56$	2.92 (1.50–5.69) 0.002 $n = 70$	2.97 (1.46–6.03) 0.003 $n = 50$	<0.0001	0.83
Hispanic ($n = 329$)	Referent $n = 32$	1.75 (0.88–3.49) 0.11 $n = 59$	2.38 (1.23–4.63) 0.01 $n = 61$	2.99 (1.53–5.85) 0.001 $n = 70$	5.19 (2.68–10.03) <0.0001 $n = 107$	<0.0001	0.30

Type 2 diabetes All ethnicities ($n = 781$)	Referent $n = 73$	1.14 (0.70–1.86) 0.61 $n = 120$	1.42 (0.86–1.86) 0.15 $n = 168$	1.71 (1.09–2.70) 0.02 $n = 182$	2.81 (1.80–4.39) <0.0001 $n = 238$	<0.0001	–
White ($n = 158$)	Referent $n = 22$	0.93 (0.46–1.92) 0.84 $n = 26$	0.81 (0.37–1.77) 0.60 $n = 28$	1.16 (0.57–2.38) 0.68 $n = 35$	1.62 (0.80–3.25) 0.18 $n = 38$	0.10	Referent
Chinese ($n = 105$)	Referent $n = 17$	0.69 (0.17–2.78) 0.60 $n = 23$	0.69 (0.18–2.61) 0.59 $n = 17$	0.67 (0.18–2.49) 0.55 $n = 17$	0.97 (0.28–3.34) 0.96 $n = 22$	0.96	0.46
Black ($n = 330$)	Referent $n = 15$	1.83 (0.75–4.44) 0.18 $n = 45$	2.01 (0.85–4.71) 0.11 $n = 74$	1.67 (0.72–3.91) 0.23 $n = 65$	4.44 (1.93–10.21) <0.0001 $n = 104$	<0.0001	0.21
Hispanic ($n = 259$)	Referent $n = 19$	1.38 (0.24–7.90) 0.72 $n = 26$	4.96 (1.09–22.6) 0.04 $n = 49$	8.93 (2.02–39.38) 0.004 $n = 65$	8.28 (1.87–36.54) 0.005 $n = 74$	<0.0001	0.09

[a]Adjusted for age, gender, waist circumference, study site, alcohol, smoking, and exercise plus ethnicity in the entire cohort
[b]*P*-value corresponds to the point estimate for odds of disease within each quintile

Table 4 Adjusted associations between GGT and metabolic risk factors, overall and in metabolic disease subgroups

	Metabolic subgroup							
Risk variable	Entire (n = 6,446) $\beta_{Adj.}$* ln (GGT)$_{(95\%CI)}$	*P*-value	Normal (n = 3,783) $\beta_{Adj.}$* ln (GGT)$_{(95\%CI)}$	*P*-value	MetS (n = 1,935) $\beta_{Adj.}$* ln (GGT)$_{(95\%CI)}$	*P*-value	Diabetes (n = 852) $\beta_{Adj.}$* ln (GGT)$_{(95\%CI)}$	*P*-value
Glucose, fasting (FBG, mg/dl)								
M1	0.11 (0.10–0.13)	<0.0001	0.03 (0.026–0.04)	<0.0001	0.04 (0.03–0.06)	<0.0001	0.13 (0.07–0.20)	<0.0001
M2	0.11 (0.10–0.13)	<0.0001	0.03 (0.02–0.04)	<0.0001	0.04 (0.02–0.06)	0.001	0.13 (0.02–0.23)	0.02
M3	0.07 (0.05–0.09)	<0.0001	0.03 (0.02–0.04)	<0.0001	0.05 (0.02–0.07)	<0.0001	0.05 (−0.05–0.15)	0.34
Insulin, fasting (FI, mU/l)								
M1	0.53 (0.49–0.57)	<0.0001	0.39 (0.34–0.44)	<0.0001	0.38 (0.31–0.45)	<0.0001	0.42 (0.28–0.55)	<0.0001
M2	0.55 (0.50–0.60)	<0.0001	0.40 (0.38–0.47)	0.0001	0.38 (0.28–0.47)	<0.0001	0.45 (0.25–0.65)	<0.0001
M3	0.39 (0.34–0.44)	<0.0001	0.33 (0.27–0.39)	<0.0001	0.38 (0.28–0.47)	<0.0001	0.47 (0.27–0.67)	<0.0001

Hemoglobin A1c (HbA1c, %)								
M1	0.06 (0.05–0.07)	0.0001	0.02 (0.008–0.02)	0.0001	0.04 (0.02–0.05)	<0.0001	0.03 (−0.01–0.08)	0.13
M2	0.06 (0.05–0.08)	<0.0001	0.02 (0.008–0.02)	<0.0001	0.02 (0.003–0.04)	0.02	0.04 (−0.02–0.11)	0.18
M3	0.04 (0.03–0.05)	<0.0001	0.01 (0.004–0.02)	0.004	0.02 (0.002–0.04)	0.03	0.04 (−0.03–0.11)	0.26
HOMA-IR								
M1	0.64 (0.60–0.69)	<0.0001	0.43 (0.37–0.48)	0.0001	0.42 (0.35–0.50)	<0.0001	0.55 (0.41–0.69)	<0.0001
M2	0.66 (0.60–0.72)	<0.0001	0.44 (0.37–0.51)	<0.0001	0.42 (0.31–0.52)	<0.0001	0.57 (0.36–0.79)	<0.0001
M3	0.47 (0.41–0.52)	<0.0001	0.36 (0.29–0.43)	<0.0001	0.42 (0.32–0.53)	<0.0001	0.52 (0.30–0.73)	<0.0001
$M4_{HOMA}$	0.37 (0.32–0.42)	<0.0001	0.28 (0.22–0.34)	<0.0001	0.39 (0.29–0.48)	<0.0001	0.43 (0.24–0.62)	<0.0001

FBG, FI, HbA1c, HOMA-IR, and GGT are log transformed. All models exclude insulin users

Regression coefficient units for FBG, FI, HbA1c, and HOMA-IR are: 10*mg glucose/unit GGT activity; mU insulin/unit GGT activity

% HbA1c/unit GGT activity and 1/unit GGT activity, respectively

M1 adjusts for age, gender, ethnicity, and study site

M2 equals M1 + alcohol use (current/former/never and drinks/week) + exercise (MET-min/week) + smoking (current/former/never and pack years)

M3 equals M2 + lipids (LDL-C, HDL-C, triglycerides), lipid-lowering medications, systolic and diastolic blood pressures, antihypertensive medications, diabetes medications, and family history of heart attack or stroke

$M4_{HOMA}$ equals M3 + waist circumference

The Postprandial State, Oxidative Stress, and Endothelial Dysfunction

Ceriello et al. have conducted trials in multiple populations, including healthy humans and patients with type 2 diabetes, demonstrating acute increases in biomarkers of oxidative stress (oxidized LDL and nitrotyrosine) and impaired flow-mediated dilation (FMD) following experimentally-induced hyperlipidemia and hyperglycemia following high fat and high glycemic index control meals (Dickinson et al. 2008; Ceriello et al. 2002, 2008; Ceriello 2000, 2003). Other example meals that have been administered as positive control meals to induce short-term reductions in FMD include fast food dishes (e.g., Big Mac®, Egg McMuffin®, hash browns), corn oil, pizza, and whipped cream (Carroll and Schade 2003; Kay and Holub 2002; Bogani et al. 2007; Esmaillzadeh et al. 2007). The control meals used by Ceriello et al. resulting in short-term endothelial dysfunction included a 70 g fat bolus as whipped cream +/− a 75 g oral glucose tolerance test or a standardized, high-fat breakfast meal (Ceriello et al. 2002). These important trials have provided detailed proof-of-concept data regarding the interrelations between metabolic status, dietary intake, postprandial oxidative stress, and subsequent acute changes in vascular response.

Glutathione Requirements for Metabolism of Dietary Iron, Lipid Peroxides, and AGE Products

Many compounds found in food, and especially cooked food, require GSH for either metabolism or elimination, providing a mechanistic rationale for how GGT may be associated with dietary characteristics. Three of these compounds include dietary iron present in mainly animal foods, lipid peroxides created as a consequence of cooking fatty acids at high temperatures, and advanced glycation end (AGE) products formed from aerobic cooking of protein and carbohydrates together.

Iron intake requires glutathione and has been associated with increased GGT activity. Serum ferritin (an iron storage protein) as well as heme iron consumption from meat have been both positively associated with GGT activity in large cohorts including the EPI-Norfolk cohort and CARDIA (Forouhi et al. 2007; Lee et al. 2004).

Lipid peroxides (LPx) induce glutathione-s-transferase (GST) and require glutathione for metabolism. Human liver microsome ex vivo and animal in vivo research has established lipid peroxidation product 4-hydroxynonal (4-HNE) induces glutathione-s-transferase, and a glutathione conjugate is formed during its metabolism (Awasthi et al. 2005; Sharma et al. 2004; Yang et al. 2003; Fukuda et al. 1997; Huang et al. 2012; Prabhu et al. 2004; Luckey and Petersen 2001; Tjalkens et al. 1999). Specific to human vascular disease, 4-HNE is known to directly increase endothelial permeability and dysfunction, and induce NRF-2 to upregulate glutathione-s-transferase (Yang et al. 2004, 2008; Siow et al. 2007; Usatyuk et al. 2006). Although the induction of NRF-2 by 4-HNE has been argued as “cardioprotection” in cardiac

myocytes (Zhang et al. 2010), sustained exposure of the endothelium to lipid peroxides and 4-HNE appears causative of endothelial dysfunction (Yang et al. 2004, 2008; Siow et al. 2007; Usatyuk et al. 2006) – an exposure we hypothesize is represented by a chronically increased fasting GGT activity compared to those with less exposure to lipid peroxides.

AGE products require glutathione for metabolism via glyoxalase enzyme activity. Metabolism to AGE products requires the availability of reduced glutathione in several known tissues, including the vascular endothelium, and the renal epithelium. Cai et al. elegantly demonstrated incubation of human endothelial cells with AGE immediately caused depletion of reduced glutathione and increase glutathione peroxidase activity (Cai et al. 2002). Notably, hepatic glutathione depletion is known to cause hepatic AGE accumulation and reduced phase 2 conjugation (Masterjohn et al. 2013) because cellular AGE metabolism is facilitated by the glyoxalase enzyme system, which also requires reduced glutathione (Wu et al. 2002).

The Relationship Between GGT and Diet

Support for the validity of GGT as a biomarker of dietary factors comes from multiple cohorts, including the TromsØ cohort study, NHANES, CARDIA, and EPI-Norfolk. The TromsØ cohort measured GGT in over 21,000 men and women and evaluated population determinants of GGT activity (Nilssen et al. 1990). Inverse associations were found between GGT activity and the consumption of coffee, which is known to increase erythrocyte glutathione levels (Esposito et al. 2003). Data from both NHANES III and CARDIA support inverse associations between dietary antioxidant intake (vitamin C, vitamin E, and beta-carotene) and increasing deciles of GGT activity. Increasing quartiles of estimated antioxidant consumption (vitamin C, vitamin E, and beta-carotene) was inversely associated with GGT activity in CARDIA, while in NHANES similar associations were found between increased *serum* concentrations of dietary antioxidant and GGT (Lee et al. 2004; Lim et al. 2007). The cumulative data support *lower* GGT activity with a dietary pattern generally considered "cardiovascularly protective" including: high intakes of vegetables, whole grains, legumes, plus raw, steamed, and poached foods, and lower intakes of refined carbohydrates, saturated fat, animal-based proteins, plus fried, grilled, and broiled foods.

Serum GGT Activity as a Postprandial Indicator

As proof-of-concept of postprandial GGT elevations, we measured GGT in 47 metabolically healthy adult volunteers following a mixed-macronutrient, cooked fast-food meal containing 1,420 kcal, including 74 g of fat (22 g saturated) and 89 g of sugar. Participants were required to consume the entire meal within 30 min. Serum was collected in the fasting state and 1, 2, and 4 h after meal administration. Figure 4a below demonstrates changes in GGT following the meal, including elevation

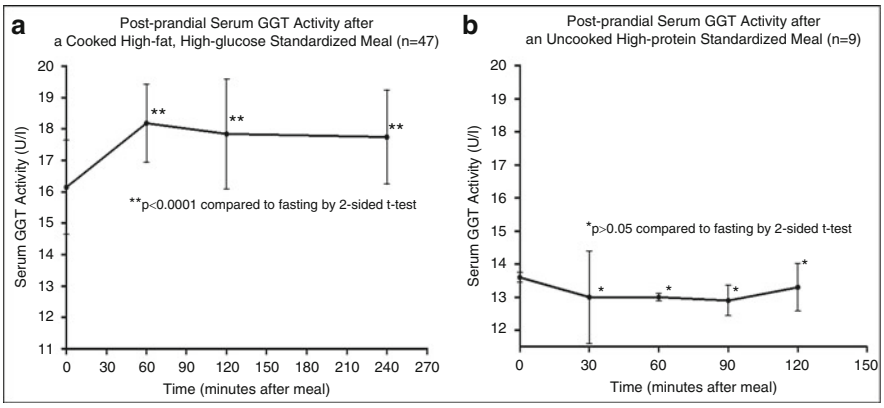

Fig. 4 (**a**, **b**) Postprandial changes in GGT. Changes in serum GGT activity acutely following: cooked high fat/glucose (**a**) versus uncooked high protein mixed-meals in healthy human volunteers (**b**)

compared to baseline persisting until at least 4-h postprandial; the total area under the curve was 371.4 U/l/t. Although these data support GGT elevations occur in the postprandial state, even in insulin-sensitive humans, we were unable to determine the dietary components responsible for this elevation because of the mixed-nutrient nature of the meal administered. We considered the postprandial increase we observed may be a generalized response to eating and therefore conducted an experiment to measure GGT activity following a very different standardized meal, i.e., a body-weight adjusted, uncooked, high-protein shake and snack bar, which led to the data in Fig. 4b, demonstrating no significant increases in postprandial GGT, and in fact a minor reduction; the total area under the curve was −61.50 U/l/t.

Ongoing and Future Research

Although the preliminary data support an interesting potential relationship between serum GGT activity and dietary characteristics – a relationship that, if proven, could provide enormous insight into the mechanisms of diet-induced cardiometabolic disease – there are several research gaps that must be filled. Three research studies are underway to determine: (1) whether acute intake of iron, lipid peroxides, and/or AGE products cause elevations in postprandial serum GGT activity; (2) whether postprandial serum GGT activity correlates to physiologically-measured endothelial function; (3) whether postprandial serum GGT activity varies in metabolic subgroups according to insulin resistance status; and (4) determine whether preprandial administration of NAC modifies any observed increases in GGT.

Our preliminary research (Fig. 4) demonstrates GGT activity increases in the postprandial state *in metabolically healthy humans* following acute intake of a cooked meal containing sugar, fat, iron, lipid peroxides, and AGE products, but does not increase significantly following an uncooked protein meal, leading to our

hypothesis that either the nutritional content of the meal itself, e.g., iron, or by products of the cooking process, i.e., lipid peroxides (LPx) and AGE products, are responsible for the observed increase. The basic science evidence for increases in GGT activity being coupled to glutathione demand in vivo and the dependency on glutathione for the metabolism of iron, lipid peroxides, and AGE products justify the need to systematically evaluate these dietary components individually to determine which components are most highly correlated to acute increases in GGT activity. Our current research is comparing a control meal low in iron, LPx, and AGE products to standardized test meals designed to differentially increase iron, LPx, and AGE products.

Our preliminary data in the MESA cohort (Table 1) demonstrates the very strong correlations between GGT activity and biomarkers of endothelial dysfunction and atherosclerosis (Bradley et al. 2014). These data combined with findings from other groups demonstrating reduced postprandial endothelial function after the acute administration of meals very similar to the meal after which we measured increased GGT activity led to our hypothesis that postprandial increases in GGT activity are concurrent with reductions in endothelial function. As endothelial dysfunction can be caused by increased oxidative stress (with subsequent decline in endothelial nitric oxide synthase product of nitric oxide), and glutathione serves as a critical keystone of in vivo antioxidant defense, it is feasible that increased GGT activity represents acute demand for glutathione in vivo, and if not available in adequate supply, endothelial function may be compromise (if only transiently). This hypothesis will be tested in proposed research by simultaneously measuring postprandial endothelial function via peripheral tonometry and then correlating observed changes between fasting and the postprandial period with the area under the GGT activity curve for the same time period. Independent of our findings related to correlations with GGT activity, the results of the proposed research will provide important data on potentially differential endothelial responses to acute intake of dietary iron, lipid peroxide, and AGE products.

Additionally, our preliminary data in the MESA cohort (Fig. 3) demonstrate the very strong correlations between GGT activity and insulin resistance state, including composite cardiometabolic risk conditions (i.e., the metabolic syndrome) (Bradley et al. 2013). Our data in MESA also demonstrate associations between GGT activity and biomarkers of atherosclerosis (Fig. 2) are attenuated with adjustment for insulin resistance (Bradley et al. 2014). These observations, combined with the findings of Ceriello et al. demonstrating that baseline oxidative stress is higher in insulin resistance participants and that postprandial reductions in endothelial function are greater in insulin resistance participants (Ceriello 2000, 2003), led to our hypothesis that the insulin resistance status of the individual may mediate the amplitude and duration of postprandial increases in GGT activity. This hypothesis will be tested by recruiting three subgroups of human volunteers who vary by degree of insulin resistance (i.e., metabolically normal, prediabetes, and type 2 diabetes) and comparing the area under the postprandial GGT activity curve for each standardized test meal compared to the control meal *within and between each participant subgroup*.

Our preliminary clinical data (Fig. 5) in humans with type 2 diabetes, and the findings of other groups, provide rational for collecting additional data on the impact

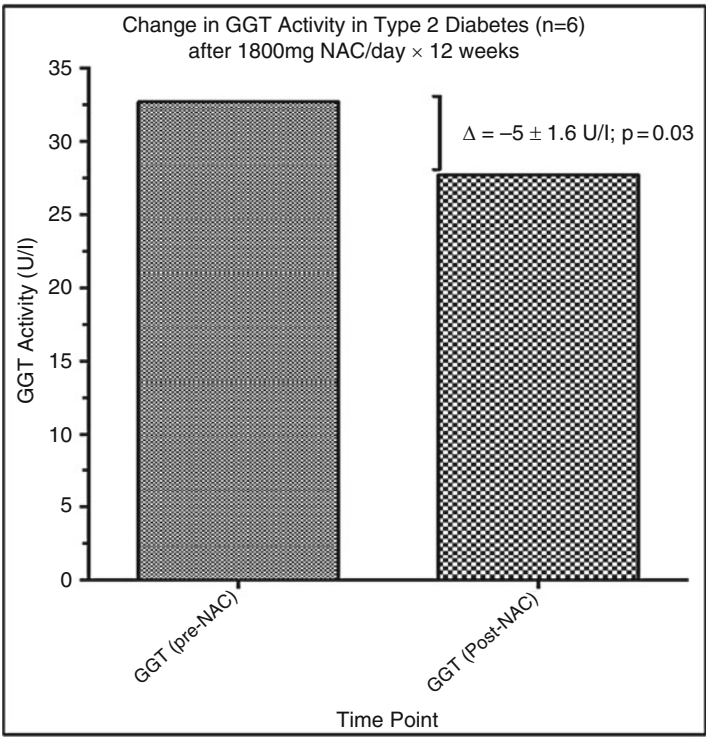

Fig. 5 Changes in GGT activity following treatment with 1,800 mg NAC in humans with type 2 diabetes ($n = 6$). Changes in fasting GGT activity in participants with type 2 diabetes, before and after treatment with 1,800 mg/day of NAC for 12 weeks

of n-acetylcysteine (NAC) on GGT activity. In GGT-knockout animals, treatment of these animals with NAC prevents the development of complications *and* prevents their premature death (Chevez-Barrios et al. 2000). Further support that GGT activity may be modified by NAC, which can contribute to clinically significant changes in metabolism, is represented by our preliminary data in people with type 2 diabetes (see Fig. 4a, b). Specific to our proposal, but untested in humans, pretreatment of human umbilical vein endothelial cells with NAC obliterated the glutathione depleting effects of AGE products (Cai et al. 2002).

Potential Application to Prognosis, Other Diseases, or Conditions

One of the most clinically relevant applications of GGT activity is its potential contribution to the identification of "fat but fit" individuals, or those patients who, despite being obese, are metabolically fit and are at lower risk for developing type

2 diabetes. This concept is supported by the research of Lim et al. in NHANES that demonstrates an important interaction between GGT, BMI, and subsequent risk of developing type 2 diabetes (Lim et al. 2007). Specifically, for those with a BMI between 30 and 34.9, the odds ratios for developing type 2 diabetes according to GGT were: GGT <22 U/l: 1.3; GGT 23–35 U/l: 7.1, and GGT >35: 15.4. For those with a BMI > 35, the odds ratios for developing type 2 diabetes according to GGT were: GGT <22 U/l: 2.4; GGT 23–35 U/l: 11.3, and GGT >35: 19.3. These findings suggest a strong interaction that could be used immediately in clinical practice to identify individuals at potentially extreme risk of developing diabetes.

Additional applications include the emerging utility of GGT subfractionation. Should GGT subfractionation become available for clinical use, the applications include: identification of alcoholism through the measurement of s-GGT, localization of tissue-specific glutathione demands (which could be used to guide route of administration of NAC or other antioxidants), and the identification of glutathione demand especially significant to cardiovascular disease through the measurement of b-GGT. Theoretically, the measurement of GGT subfractions could be used very similarly to current isozyme measurement of other routine clinical lab measures, like alkaline phosphatase, in order to identify its tissue of origin.

Conclusion

The data presented in this chapter, combined with the accumulation of past evidence, support total serum GGT activity as a significant biomarker of cardiovascular and metabolic risk, including strong associations with established individual risk factors for vascular disease, plus composite risk conditions including metabolic syndrome and type 2 diabetes. The established role of GGT in GSH homeostasis supports the need for translational investigations of the behavioral and environmental causes of increased GGT activity in humans, including triggers, regulators, and more precise understanding of its relationship to GSH status in single- and multiorgan pathology.

Our findings in the MESA cohort support the hypothesis that increases in serum GGT activity occur in parallel with increases in oxidative stress, immune activation, acute phase response, and vascular inflammation, and serum GGT activity may represent the influence of metabolic status on vascular injury. These results are consistent with in vivo findings that increased GGT activity represents a reduction in available GSH (Sedda et al. 2008), potentially leaving substrates, e.g., LDL, vulnerable to oxidative modification, which then activate immune targets. Based on the strength and consistency of the associations demonstrated here, combined with current understanding of the oxidative/inflammatory mechanisms of endothelial dysfunction, we conclude serum GGT activity should be considered a continuous biomarker of increased glutathione demand relevant in the development of endothelial dysfunction and subsequent arteriosclerosis.

Although more experimental research is needed, the available research supports a relationship between GGT elevation and dietary patterns long known to be associated with cardiovascular disease. Our group and others are closer to determining dietary characteristics which directly influence GGT activity, which may provide a pathway to more precise research regarding diet-induced "oxidative stress" and the relative contributions of different food types and preparation methods to cardiovascular disease.

Given the importance of redox balance to cellular metabolism in all tissues, it is conceivable that total GGT activity is a biomarker of multiorgan GSH depletion, whereas increases in tissue-specific GGT activity subfractions suggested chronic tissue-specific GSH depletion. Extending this research may even result in the ability to differentiate isolated liver, kidney, vascular disease, as well as, chronic disease with multiple affected organs, i.e., type 2 diabetes. If this differentiation is confirmed by additional experimental research, it would fill an important research gap in population-based cohort studies (Mayne 2003).

The low cost of measuring GGT and its stability in stored samples makes it ideal for continued confirmatory research in large population-based cohort studies. Yet, given the accumulation of population-based research findings suggesting cross-sectional associations with established cardiometabolic risk factors, as well as increased cardiometabolic event prediction from graded elevations in GGT activity, we suggest translational clinical research is now needed to determine the mechanistic determinants of serum GGT activity.

Summary Points

- Increased GGT activity is associated with riskier cardiovascular risk profile, including male gender, smoking, increased alcohol use, hyperlipidemia, hypertension, and insulin resistance.
- Literature supports both epidemiological associations between increased GGT activity (within the normal range) and cardiovascular events, as well as mechanistic biomarkers like C-reactive protein, vascular adhesion molecules, inflammatory cytokines, and oxidized LDLs.
- GGT activity is associated with metabolic risk, including individual risk factors, including elevated BMI and waist circumference, lifestyle factors, as well as clinical risk factors including hemoglobin A1c, fasting glucose, and fasting insulin.
- The odds of prevalent metabolic syndrome and type 2 diabetes increases with elevations in GGT activity.
- Dietary components requiring glutathione for metabolism, including iron, lipid peroxides, and advanced glycation end (AGE) products, are candidate exposures to evaluate for the acute induction of GGT activity and its relationship to atherosclerosis.

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Plasma Factor VIII Levels as a Biomarker for Venous Thromboembolism

30

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Contents

Abstract

Venous thromboembolism (VTE) is a multicausal disease. Multiple genetic, acquired, and environmental factors contribute to the development of the disease, and most of these factors are related to changes in flow and composition of the

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_22

blood, leading to a hypercoagulable state. Increasing data support the hypothesis that high levels of factor VIII (FVIII) in plasma may play a role in hypercoagulability. An association between increased plasma FVIII levels and VTE was first described in the Leiden Thrombophilia Study. Subsequently, these findings have been supported by several independent case-control studies. Cumulatively, these studies have clearly demonstrated that high FVIII levels constitute a prevalent, independent, and dose-dependent risk factor for the first episode of VTE. Furthermore, subsequent studies have shown that the risk of a recurrent episode is also significantly increased in patients with high FVIII levels. However, the molecular basis of high plasma FVIII levels is incompletely known. In this chapter, we discuss the biological mechanisms that may underlie persistently elevated FVIII levels and the pathways through which high FVIII may increase the thrombotic risk. In addition, we present and review evidences supporting the hypothesis that elevated plasma FVIII levels constitute an important biomarker for VTE risk.

Keywords

Factor VIII • Venous thromboembolism • Recurrence • Risk factor • Coagulation • Post-thrombotic syndrome

Abbreviations

APTT	Activated partial thromboplastin time
Ca^{2+}	Calcium
CHARGE	Cohorts for Heart and Aging Research in Genomic Epidemiology
CRP	C-reactive protein
DVT	Deep venous thrombosis
ESR	Erythrocyte sedimentation rate
FII	Prothrombin
FIIa	Thrombin
FIX	Factor IX
FV	Factor V
FVII	Factor VII
FVIII	Factor VIII
FX	Factor X
FXI	Factor XI
FXII	Factor XII
LETS	The Leiden Thrombophilia Study
LRP1	Low-density lipoprotein receptor-related protein 1
mRNA	Messenger ribonucleic acid
NE	Not evaluated
PE	Pulmonary embolism
PTS	Post-thrombotic syndrome
TF	Tissue factor
VTE	Venous thromboembolism
VWF	von Willebrand factor

Key Facts of High Factor VIII Levels

- The coagulation cascade is essential for the coagulation system and has been studied for many decades.
- The factor VIII is an important compound of coagulation cascade.
- Factor VIII deficiency is associated to a severe bleeding disorder called hemophilia A.
- High plasma factor VIII levels are associated with thrombotic disorders, as venous thromboembolism.
- Venous thromboembolism is a condition in which a blood clot forms in one or more veins, causing their total or partial obstruction.
- Venous thromboembolism is presented as superficial venous thrombosis, deep venous thrombosis, or pulmonary embolism.

Definitions

Acute phase response It is a response of the body to a tissue injury.

Coagulation cascade It is a complex sequence of chemical reactions resulting in the formation of a fibrin clot.

Deep venous thrombosis It is a condition in which blood clot forms in one or more deep veins of the body, mainly in legs.

Factor VIII It is a key protein for the coagulation cascade.

Post-thrombotic syndrome It is a common and chronic complication of deep venous thrombosis of the legs.

Pulmonary embolism It is a condition in which blood clot forms in a pulmonary vein.

Recurrent thrombosis It is novel episodes of thrombosis after an initial thrombotic event.

Thrombophilia It is the tendency to develop thrombosis blood clots due to an abnormality in the coagulation system.

Tissue factor It is the trigger for the coagulation cascade.

Venous thromboembolism It is a condition in which a blood clot forms in one or more veins and comprises deep venous thrombosis (DVT) and pulmonary embolism (PE).

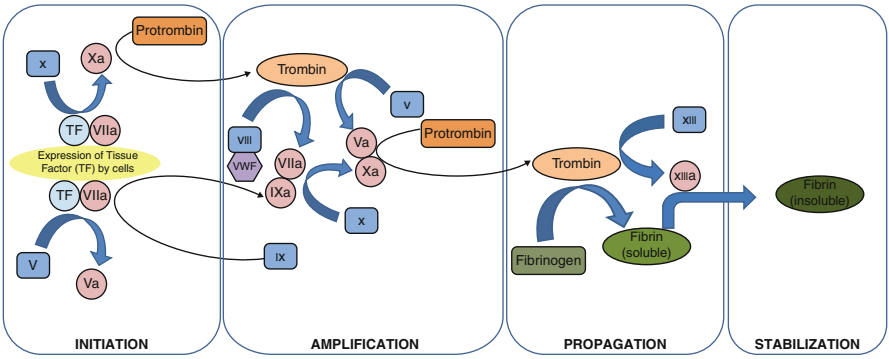

Fig. 1 Classic representation of the coagulation cascade, including its four pathways (initiation, amplification, propagation, and stabilization)

Introduction

The hemostatic system is a complex network of cells, proteins, and other molecules needed for bleeding control. In physiological conditions, this system allows the circulation of blood, while thrombus formation occurs only following damage to the vascular endothelium. The hemostatic system requires tight regulation: uncontrolled activation of the hemostatic system may result in a hypercoagulable state leading to the occlusion of the vascular system, a condition known as thrombosis. On the other hand, if the hemostatic system fails to react appropriately upon injury, an unstoppable hemorrhage might occur (Wolberg et al. 2012).

The hemostatic system is basically composed of five components: endothelial cells, platelets, coagulation cascade, natural anticoagulants, and fibrinolysis. The primary response of the hemostatic system involves the vascular endothelium and platelets, resulting in the formation of platelet thrombi. The coagulation cascade produces fibrin, which will strengthen and stabilize this primary thrombus. Finally, the fibrinolysis will gradually dissolve the thrombus to restore the normal blood flow. The coagulation cascade, in particular one of its components, factor VIII (FVIII), is the object of discussion in this chapter (Wolberg et al. 2012).

The coagulation cascade is essential for the coagulation system and has been studied for many decades. Physiologically, it can be divided into four pathways: initiation, amplification, propagation, and stabilization (see Fig. 1). The initiation pathway produces small amounts of thrombin that activates a variety of components that allows amplification of the cascade to produce large amounts of thrombin. Thrombin is a key protein that cleaves fibrinogen to fibrin resulting in a stable clot formation (Lillicrap 2008).

Briefly, the initiation pathway is triggered by the exposure of endothelial tissue factor (TF) to blood circulation. When exposed by subendothelial cells, TF binds to circulating FVII, which is activated into FVIIa. The complex FT-FVIIa is responsible for the activation of FIX and FX, and FXa is then responsible for the conversion

of prothrombin into small amounts of thrombin. The so-called amplification pathway occurs when the small amounts of thrombin activate, on the surface of platelets, FV, FVIII, and FXI, and then FXIa activates FIX. The propagation pathway involves the formation of two proteic complexes: first, the tenase complex FIXa-FVIIIa, which activates FX, and, second, the prothrombinase complex FXa-FVa, which is responsible for the generation of large amounts of thrombin. Thrombin, in larger amounts, converts fibrinogen to fibrin, the protein responsible for the establishment of a stable blood clot (Lillicrap 2008).

Factor VIII and Venous Thromboembolism

Factor VIII

FVIII is an important compound of coagulation cascade. Upon injury within the vasculature, the proteolytic cascade of coagulation activates the cofactor FVIII to FVIIIa, resulting in its dissociation from von Willebrand factor (VWF). On the platelet surface, FVIIIa is assembling with an enzymatically active form of FIX (FIXa) to form a macromolecular tenase complex. This complex effectively activates FX, the next proenzyme in the coagulation cascade, to FXa. The major organ of FVIII production is the liver; however, the presence of FVIII mRNA observed in other organs, such as the spleen, lungs, kidneys, and brain, suggests that these tissues may also contribute to the circulating FVIII levels (Wion et al. 1985; Hollestelle et al. 2001).

Immediately after its release into the circulation, FVIII is caught into a tight non-covalent complex with its carrier, the VWF, which has two major functions. First, VWF works as a "bridge" between the damaged endothelium and the platelets. Second, VWF acts as a natural carrier of FVIII, and the complex VWF/FVIII is crucial for the survival of FVIII in the blood circulation by its protection against improper proteolytic activation and premature clearance. Plasma FVIII levels are influenced by many factors, including plasma level of VWF, ABO blood type, age, ethnicity, and polymorphisms in genes related to FVIII (Lenting et al. 2007, 2010; Lillicrap 2008).

The importance of FVIII for the coagulation system is illustrated by the severe bleeding disorder called hemophilia A, caused by its absence or decrease in plasma. On the other hand, several epidemiological studies have also showed an association between elevated FVIII levels and thrombotic complications. This chapter will focus on the research results regarding the association of high FVIII levels and venous thrombosis, in the last two decades.

Venous Thromboembolism

Venous thromboembolism (VTE) is a condition in which a blood clot forms in one or more veins and comprises deep venous thrombosis (DVT) and pulmonary embolism

(PE). According to Virchow's triad, VTE may be caused by bloodstream stasis, vascular wall injuries, or blood thickening (Wolberg et al. 2012). Thrombophilia is defined as a tendency to VTE due to the presence of inherited or acquired risk factors. VTE is a multifactorial disease, and, generally, it is a result of the interaction between inherited and acquired risk factors (Rosendaal 1999b).

The known acquired risk factors for VTE are immobilization, pregnancy, puerperium, cancer, the use of estrogen hormones, trauma, surgery, and the presence of antiphospholipid antibody. Inherited thrombophilia occurs due to genetic defects that compromise the natural anticoagulation mechanisms (such as protein C, protein S, and antithrombin deficiencies) or that lead to a procoagulant state (such as the G1691A mutation in the factor V gene – factor V Leiden – or the G20210A mutation of the prothrombin gene) (Rosendaal 1999a; Robertorye and Rodgers 2001). From studies of familial thrombophilia, we also know that there are still other genetic risk factors that we have not identified yet (De Visser et al. 2013; Zöller et al. 2014).

Primarily, the diagnosis of an acute DVT or PE episode may be performed by a variety of imaging exams, such as duplex ultrasonography, computed angiotomography, and ventilation-perfusion scanning. However, plasma analyses have been a very useful tool, since some molecules are indentified as biomarkers of VTE diagnosis or recurrence, including D-dimer and FVIII levels (Meijer and Schulman 2009; Hou et al. 2012). VTE treatment consists of anticoagulant therapy. As VTE represents an important health problem worldwide, because of the economic, social, and health burden impact, biomarkers capable to identify the population with a higher risk to develop this disease could be very useful for the management of this condition.

Methods to Evaluate Plasma FVIII Levels

Pre-analytical Guidelines

A procedural guideline for the collection, transport, and storage of plasma-based coagulation assays is necessary, as many pre-analytical variables may affect the results of FVIII activity assay.

Collection: It is recommended that blood samples for coagulation assays (including FVIII activity assay) be collected by venipuncture using a blood collection system that collects the sample directly into a glass or plastic evacuated tube containing the appropriate anticoagulant. Blood samples can also be collected into a syringe, but it is important that the blood is added to the appropriated tube within 1 min of completion of draw. For both methods of collection, it is crucial to mix gently the tubes with blood sample immediately at the end of collection procedure. The anticoagulant recommended for FVIII activity assay should be 3.2 % of the dihydrate form of trisodium citrate. Moreover, the proportion of blood to the anticoagulant is 9:1 and must be respected. An inadequate filling of the tube will decrease this ratio and may compromise the results (CLSI 2008).

Transport: Plasma samples for FVIII activity assay should be transported at room temperature (transportation on ice is not recommended). Extreme temperatures during transportation must be avoided. Moreover, the transport to processing laboratory should be as fast as possible, but may occur within 4 h from the collection (CLSI 2008).

Storage: The temperature of storage depends on the time between the storage and analysis. For FVIII activity assay, the plasma sample is stable at −20 °C for 2 weeks and at −80 °C for 18 months. Importantly, as precipitation of some proteins may occur with freezing, the plasma sample mixing is critical before testing (CLSI 2008).

FVIII Activity Assays

Different methods have been devised for the determination of FVIII coagulant activity, including the one-stage and two-stage clotting assays and the chromogenic assay. The one-stage clotting assay is based on the ability of test samples to correct the activated partial thromboplastin time (APTT) of a FVIII-deficient substrate plasma. The FVIII-deficient plasma and patient sample are preincubated with the APTT reagent which contains a contact activator (e.g., ellagic acid, kaolin, silica, celite) and phospholipid. Calcium chloride is added to promote fibrin clot formation, the measured end point of the APTT. FVIII concentration in the patient sample is assumed to be the rate-limiting determinant of the clotting time. The result is compared with a standard curve generated from samples containing known FVIII activities. Wherever possible, the specificity of the deficient plasma should be checked to ensure that there is a single FVIII deficiency. The FVIII-deficient plasma should have normal levels of the non-deficient factors and <1 IU/dL of the FVIII (Mackie et al. 2013; Gouws et al. 2014).

Although the one-stage method has been preferred in the routine diagnostic because of the simplicity of the method, as well as the wide availability of reagents and the ease of automation, the two-stage method was considered to be the reference method. The main reason for this was the propensity of interference by substances like lipids and heparin in the one-stage method, which did not seem to occur in the two-stage method. Nowadays, this reference method was replaced by the chromogenic method which also shows no interference by heparins, direct thrombin inhibitors, or lupus anticoagulants (Mackie et al. 2013; Gouws et al. 2014).

In the chromogenic assay, patient plasma is added to a reaction mixture consisting of thrombin or prothrombin, FIXa, FX, calcium, and phospholipid. This nearly immediately produces FVIIIa, which works with FIXa to activate FX. When the reaction is stopped, FXa production is assumed to be proportional to the amount of functional FVIII present in the plasma sample. FXa measurement is made through cleavage of an FXa-specific chromogenic substrate, and the product of this reaction produces a color that can be measured photometrically by absorbance at 405 nm. The color produced is directly proportional to the amount of functional FVIII present in the plasma sample based on a standard curve. The limitations of the chromogenic assay are the limited number of commercially available assay kits and the high cost when compared with the one-stage clotting assay (Gouws et al. 2014).

Despite the chromogenic and one-stage method, clot-based methods seem to be well correlated; discrepant results were reported in studies of patients with hemophilia A. Therefore, the current consensus seems to utilize both methods for the diagnosis of hemophilia A (Trossaërt et al. 2011; Potgieter et al. 2015). Elevated levels of FVIII are associated to a thrombotic risk. Determining levels in this patient population is easier and holds less analytic pitfalls in chromogenic and one-stage clot-based methods, showing a good correlation coefficient between both methods. However, despite this, some authors argue that the chromogenic methods hold some advantage as the assay of choice as it shows better precision and fewer interferences as compared to one-stage clot-based method (Chandler et al. 2003; Legnani et al. 2004; Gouws et al. 2014).

Factor VIII Levels as a Risk Factor for Venous Thromboembolism

High plasma FVIII levels are assumed to be a risk factor for thrombosis, with a greater impact on venous than on arterial thrombosis. The first studies on a possible association between high FVIII levels and thrombotic disease were reported on arterial disease from the early 1960s (Egeberg 1962). Since then, several case-control studies have reported the association of high FVIII levels with arterial thrombosis (Rice and Grant 1998; Tracy et al. 1999; Rumley et al. 1999; Folsom et al. 1999).

The Leiden Thrombophilia Study (LETS) was the first to report an association between high FVIII levels and VTE (Koster et al. 1995). This case-control study included 301 patients with first VTE episode and 301 healthy controls matched for age and gender. Although the median time interval between acute thrombosis and FVIII activity measurement was 18 months (minimum of 6 months), elevated FVIII levels (>150 UI/dL) were present in 25 % of the patient cohort, compared to 11 % of controls. Univariate analysis demonstrated that FVIII activity, VWF antigen, and non-O blood group were all associated with increased risk for VTE. However, on multivariate analysis, only FVIII level was a significant independent risk factor, with an odds ratio of 4.8 (95 % confidence interval 2.3–10.0). Since that, the high prevalence of elevated FVIII levels in patients with VTE has been confirmed in a number of subsequently cohort and case-control studies (Bloemenkamp et al. 1999; Kamphuisen et al. 1999; Kraaijenhagen et al. 2000; Patel et al. 2003; Oger et al. 2003; Legnani et al. 2004; Mello et al. 2009; Ota et al. 2011; Ryland et al. 2012; Table 1).

In a large prospective study, Tsai et al. (2002) measured FVII, FVIII, VWF, fibrinogen, and C-reactive protein (CRP) levels, in blood samples obtained from 19,237 adults with no history of VTE. After a median follow-up time of 7.8 years, a total of 159 VTE episodes occurred. FVIII and VWF levels were linearly associated with increased risk VTE. In contrast, there was no association of CRP or fibrinogen levels with VTE risk. Two additional reports showed dose-dependent correlation between FVIII levels and VTE risk. The authors showed that for each 10 IU/dL increased in FVIII level, the risk of first VTE increased by 10 %, and the risk of recurrent VTE increased by 24 % (Kraaijenhagen et al. 2000; Patel et al. 2003; Ota

Table 1 Case-control studies of high FVIII levels in patients with confirmed venous thromboembolism

Study	Patients (N)	Controls (N)	Time sampling	High-FVIII (>150 IU/dL) patients/ controls	Odds ratio	Dose-response effect
Koster et al. 1995	301	301	18 (6–48) months of VTE[a]	25 %/11 %	6.2	Yes
Bloemenkamp et al. 1999	155	169	≥3 months after anticoagulation	36 %/17 %	4.0	Yes
Kamphuisen et al. 1999	474	474	18 (6–48) months of VTE[a]	24 %/10 %	6.7	Yes
Kraaijenhagen et al. 2000	65	60	≥6 months of VTE	32.3 %/23.3 %	4.0	Yes
Patel et al. 2003	100	100	≥6 weeks after anticoagulation	81 %/38 %	7.0	Yes
Oger et al. 2003	161	239	At initial presentation	NE/NE	2.4	Yes
Mello et al. 2008	175	175	≥3 months of VTE	37 %/9.7 %[b]	5.3	NE
Ota et al. 2011	68	40	≥3 months of VTE	34 %/2 %	32.7	Yes
Ryland et al. 2012	91	52	7 (2–120) months after anticoagulation[a]	40 %/NE	NE	NE

This table summarizes the results of previous studies that evaluated plasma FVIII levels as a risk factor for venous thromboembolism
VTE venous thromboembolism, *NE* not evaluated
[a]Results expressed in median (range)
[b]High FVIII levels defined as >212 IU/dL

et al. 2011). These were an important data which indicate that high FVIII level may be a prevalent, independent, and dose-dependent risk factor for VTE.

FVIII Levels and VTE Recurrence

Some studies have reported the effect of FVIII levels on the recurrence of VTE, and they all seem to indicate that VTE patients with high FVIII levels (measured several months after the last VTE episode) have an increased risk of a recurrent event when compared to those with lower FVIII levels. In a retrospective study, Kraaijenhagen et al. (2000) included 60 patients with recurrent VTE, 65 patients with a single episode of thrombosis, and 60 age- and sex-matched controls. The time between acute thrombosis episode and blood drawn was at least 6 months. FVIII levels above 175 IU/dL were observed in 10 % of controls, 19 % of patients with single VTE, and 33 % of the recurrent VTE patients. Simultaneously, Kyrle et al. (2000) studied 360 patients for an average follow-up period of 30 months after the first episode of VTE, and they obtained the same conclusion via exploring the relationship between high plasma levels of FVIII and the risk of recurrent VTE. The authors showed that recurrent VTE developed in 38 of the 360 patients (10.6 %). Patients with recurrence had higher FVIII levels than those without recurrence (182 vs. 157 IU/dL).

Thus far, the hypothesis that high plasma FVIII levels may influence the risk for recurrent VTE was further showed in prospective studies (Legnani et al. 2004; Tirado et al. 2005; Cosmi et al. 2008). On the other hand, the prospective follow-up of LETS study did not find an association between high FVIII levels and risk of recurrence (Christiansen et al. 2005). Thus, despite the strong association between high FVIII levels and VTE recurrence risk in several studies, more data are needed, in special from prospective studies, before we can consider elevated FVIII levels as an established risk factor for a recurrent VTE (Table 2).

Table 2 High FVIII levels as a risk factor for recurrent venous thromboembolism

Study	Total patients (N)	Recurrent patients (N)	Definition of high FVIII levels	Odds ratio
Kraaijenhagen et al. 2000	125[a]	60	>175 IU/dL	22.5
Kyrle et al. 2000	360	38	>234 IU/dL	6.7
Legnani et al. 2004	564	53	>294 IU/dL	6.2
Tirado et al. 2005	250	65	>232 IU/dL	2.6
Christiansen et al. 2005	474	90	>166 IU/dL	No risk
Cosmi et al. 2008	336	55	>242 IU/dL	2.8

This table summarizes the description of study design and results of studies that evaluated high FVIII levels as a risk factor for recurrent VTE

VTE venous thromboembolism

[a]They were not consecutive patients

The mechanism by which FVIII levels influence thrombotic risk has not yet been identified. One important possibility is the increase in factor IXa cofactor activity, which results in an increase of FXa and thrombin formation. Two studies investigate the possible association between high FVIII levels and increased thrombin formation and showed that basal thrombin generation was significantly higher in VTE patients with high FVIII levels when compared to VTE patients with normal FVIII levels. Overall FVIII levels were strongly correlated with thrombin generation (O'Donnell et al. 2001; Ryland et al. 2012).

Despite the fact that high FVIII level is a well-defined risk factor for VTE, the reasons of this elevation are still unclear. Herein, we will discuss the role of genetics and acute phase response, probably important players in this context.

Mechanisms of Elevated FVIII Levels: The Role of the Acute Phase Response

Since FVIII protein is known to rise in response to an acute phase reaction, it is difficult to determine whether the increased FVIII levels seen in VTE patients precedes the thrombosis or represents a secondary inflammatory phenomenon. Thus, previous studies evaluated the role of acute phase response in the maintenance of elevated FVIII levels after the thrombotic episode. Accumulating evidences suggest that elevated FVIII levels cannot be explained simply as a post-thrombotic acute phase phenomenon. First, in most cohort studies, samples for FVIII analysis were drawn several months following the acute VTE. Furthermore, longitudinal follow-up studies have clearly demonstrated that the increased plasma FVIII levels observed in VTE patients tend to persist for many years (Kraaijenhagen et al. 2000; O'Donnell et al. 2000; Kyrle et al. 2000; Legnani et al. 2004; Tirado et al. 2005; Bittar et al. 2013).

Second, several studies evaluated the high FVIII levels in VTE patients in parallel with inflammatory markers as CRP, fibrinogen, and erythrocyte sedimentation rate (ESR) to quantify the extent and duration of the post-VTE acute phase response and its influence on FVIII levels. Using this strategy, despite the fact that VTE patients present higher levels of inflammatory markers when compared with healthy controls, clear inflammation was observed in only 10–18 % of patients. Moreover, plasma FVIII levels did not correlate with CRP, ESR, or fibrinogen levels (O'Donnell et al. 1997, 2000; Kamphuisen et al. 1999; Kraaijenhagen et al. 2000; Bittar et al. 2013; Table 3).

In a recent study, Tichelaar et al. (2012) investigated the time courses of FVIII, CRP, and fibrinogen levels in VTE patients immediately following the acute event. Unsurprisingly, plasma FVIII, CRP, and fibrinogen levels were increased in the majority of patients (88 %, 76 %, and 64 %, respectively) at their initial presentation (start of treatment). However, although FVIII levels remained persistently elevated in 72 % of subjects for at least 6 months, plasma CRP and fibrinogen levels had both corrected to the normal range by 3 months in the majority of patients. Therefore, these results clearly demonstrate that although systemic inflammation may be present in some VTE patients, other more important unidentified factors are responsible for long-term maintenance of elevated FVIII levels.

Table 3 The influence of inflammatory markers on FVIII levels in patients with previous VTE

Study	Patients (N)	Time sampling	Inflammatory markers	Influence on FVIII levels
O'Donnell et al. 1997	66	≥3 months of VTE	CRP; fibrinogen; ESR	None
Kamphuisen et al. 1999	474	≥6 months of VTE	CRP; fibrinogen	None
Kraaijenhagen et al. 2000	125	≥6 months of VTE	CRP; fibrinogen	None
O'Donnell et al. 2000	35	≥3 months of VTE	CRP; fibrinogen	None
Tichelaar et al. 2012	75	At initial presentation	CRP; fibrinogen	Weak
Bittar et al. 2013	55	≥8 years of VTE	CRP	None

This table summarizes the description of study design and results of studies that evaluated the influence of acute inflammation markers on FVIII levels in patients with previous venous thromboembolism

VTE venous thromboembolism, *CRP* C-reactive protein, *ESR* erythrocyte sedimentation rate

Genetics of Elevated FVIII Levels

The molecular basis of high FVIII levels is only partially known and consists of genetic and acquired factors. Blood group and VWF levels are important genetic factors that explain only partially the variation in FVIII levels. Previous studies showed that FVIII levels are at least partially determined genetically (Kamphuisen et al. 1998; Schambeck et al. 2001; Bank et al. 2005), reporting that FVIII levels show a familial clustering, which remains after correction for VWF levels and ABO blood group.

In a retrospective study of 177 patients with high plasma FVIII levels, Bank et al. (2005) observed that 40 % of their first-degree relatives also had elevated FVIII levels (>150 IU/dL). These first-degree relatives with elevated FVIII levels also demonstrated an increased risk for both VTE and arterial thrombosis when compared to first-degree relatives with normal FVIII levels. Moreover, in relatives with elevated FVIII, the absolute annual incidence for VTE was 0.34 % compared to only 0.13 % in relatives with normal FVIIII levels. Furthermore, the risk of VTE was highest in those first-degree relatives with highest plasma FVIII levels [OR 3.7 (95 % CI 1.9–7.5)].

The evidence that high FVIII levels may be genetically determined has prompted investigation of the *F8* gene in the last decade. Therefore, several studies investigated the *F8* gene, and even with the identification of several mutations and single nucleotide polymorphisms, none showed correlation with elevated FVIII levels nor VTE risk (Mansvelt et al. 1998; Kamphuisen et al. 2001; Endler and Mannhalter 2003; Morange et al. 2005; Viel et al. 2007; Ay et al. 2011; Bittar et al. 2015b). However, this does not preclude the possibility that unidentified regulatory *F8* gene sequences may play a role in this context. Thus, some studies have focused on other candidate genetic loci that may be involved in regulating plasma FVIII levels. The

Table 4 Genetic studies in *LRP1* gene and their association with FVIII levels and VTE risk

Study	Patients (N)	Controls (N)	Molecular alterations investigated	Influence on FVIII levels	Influence on VTE risk
Morange et al. 2005	0	424	C766T	None	NA[a]
			A775P	None	NA[a]
			D2080N	Decreased levels	NA[a]
Cunningham et al. 2005	60	80	C766T	None	Protection effect
			A775P	None	None
			D2080N	None	None
Marchetti et al. 2006	200	0	C-25G	Decreased levels	NA[b]
Vormittag et al. 2007	152	198	C663T	Increased levels	Risk factor
Mello et al. 2008	249	366	C220T	None	None
			A775P	NA[c]	NA[c]
			D2080N	Decreased levels	None
Bittar et al. 2015b	75	74	C2767T	None	None
			T780I	None	None

This table summarizes the molecular alterations previously investigated in *LRP1* gene in patients with venous thromboembolism

VTE venous thromboembolism, *NA* Not applicable

[a]Not applicable because no patient group was evaluated

[b]Not applicable because no control group was evaluated

[c]A775P was identified in neither patients or controls

major candidate was the low-density lipoprotein receptor-related protein 1 (LRP1), a cell surface endocytic receptor expressed on a variety of human tissues. In vitro studies showed that LRP1 can mediate cellular uptake and degradation of FVIII (Lenting et al. 1999; Saenko et al. 1999). Moreover, in vivo animal models demonstrated that plasma FVIII levels were significantly increased in LRP1-deficient mice when compared to wild-type mice (Bovenschen et al. 2003, 2005).

Following this context, several groups investigated the role of known *LRP1* gene polymorphisms on plasma FVIII levels and VTE risk in humans, and although they had evaluated several polymorphisms in this gene, most of them did not correlate with increased plasma levels of FVIII or VTE risk (Morange et al. 2005; Cunningham et al. 2005; Marchetti et al. 2006; Mello et al. 2009; Bittar et al. 2015b). On the other hand, the C663T (A217V) polymorphism of *LRP1* gene was the only molecular alteration associated with elevated FVIII levels and VTE risk (Vormittag et al. 2007; Table 4).

Recently, some genome-wide association studies have sought to identify novel genetic loci that contribute to the heritability of plasma FVIII levels (Smith et al. 2010; Antoni et al. 2011). For example, the large CHARGE (Cohorts for

Heart and Aging Research in Genomic Epidemiology) consortium study, which included more than 23,000 participants, identified six novel candidate genes associated with plasma FVIII or VWF levels (Smith et al. 2010). However, the molecular mechanisms through which these genes influence plasma FVIII and VWF levels have not yet been elucidated. Therefore, the contributions from genetic factors for the etiology of persistently elevated plasma FVIII levels need to be clarified.

Potential Applications to Prognosis, Other Diseases or Conditions of Factor VIII Levels, and Post-thrombotic Syndrome

Post-thrombotic syndrome (PTS) is a common chronic complication of DVT of the lower limbs. Patients with PTS may experience pain, heaviness, swelling, cramps, itching, or numbness in the affected limb. Symptoms can be intermittent or persistent, and tend to become worst with standing or walking, and improving with rest and leg elevation. Clinical signs include edema, telangiectasia, hyperpigmentation, varicose veins, and venous eczema. In more severe cases, ulcers may appear, and these tend to be chronic, painful, and difficult to heal, and recurrences are common (Prandoni and Kahn 2009; Kahn 2011; Table 5).

Table 5 Signs and symptoms of post-thrombotic syndrome evaluated by the Villalta scale

Signs and symptoms	Absence	Mild	Moderate	Severe
Symptoms				
Pain	0 points	1 point	2 points	3 points
Cramps	0 points	1 point	2 points	3 points
Heaviness	0 points	1 point	2 points	3 points
Paresthesia	0 points	1 point	2 points	3 points
Pruritus	0 points	1 point	2 points	3 points
Signs				
Pretibial edema	0 points	1 point	2 points	3 points
Skin induration	0 points	1 point	2 points	3 points
Hyperpigmentation	0 points	1 point	2 points	3 points
Redness	0 points	1 point	2 points	3 points
Venous ectasia	0 points	1 point	2 points	3 points
Pain during calf compression	0 points	1 point	2 points	3 points
Venous ulcer	Absence	Presence		

This table shows the PTS assessment by the Villalta scale. The scale consists of five patient-rated venous symptoms and six clinician-rated physical signs, which are each rated on a 4-point scale (0 = none, 1 = mild, 2 = moderate, 3 = severe). Points are summed to produce a total score (range, 0–33). Subjects are classified as having PTS if the score is $\geq$5 or the presence of venous ulcer. Among patients with PTS, a Villalta score of 5–9 is consistent with mild PTS, a score of 10–14 is consistent with moderate PTS, and a score of $\geq$15 or the presence of venous ulcer is consistent with severe PTS

PTS post-thrombotic syndrome

Even after an appropriate anticoagulant treatment, PTS develops in about 20–50 % of patients with DVT of the lower limbs, and 5–10 % of patients develop severe PTS (Kahn 2011). PTS is associated with an increased risk for DVT recurrence (Stain et al. 2005), poor quality of life (Smith et al. 2000), and significant cost to healthcare system (MacDougall et al. 2006). Patients with PTS have a lower quality of life compared to patients with chronic lung disease, diabetes, or arthritis, and those with severe PTS have a quality of life comparable to patients with heart failure or cancer.

The role of coagulation factors such as FVIII in the occurrence of PTS is still unclear and controversial. Goldenberg et al. (2004) showed an association between high levels of FVIII and occurrence of PTS in children. However, other studies showed no association between elevated FVIII levels and PTS (Stain et al. 2005; Kreuz et al. 2006; Bouman et al. 2012; Lyle et al. 2013; Bittar et al. 2015a). Recently, Bittar et al. (2013) reported a study that measured FVIII levels after 10 years from the index DVT and found that patients with severe PTS had higher FVIII levels. However, the selected study population was known for having a previous elevated FVIII, measured 7 years before. Thus, a possible association between elevated FVIII levels and PTS remains to be clarified.

Conclusive Remarks and Perspectives

High plasma FVIII levels are a common finding in patients with VTE and constitute an independent and dose-dependent risk factor for VTE. These findings have been consistently replicated across several independent case-control studies. Despite significant advances in the field, current knowledge still fails to identify factors underlying the long-term maintenance of elevated FVIII levels in VTE patients. Consequently, it is not clear whether elevated FVIII levels represent an inherited or an acquired thrombophilia or, rather, represent a marker of ongoing post-thrombotic acute phase response. Further studies will be required to elucidate the molecular mechanism(s) underlying high plasma FVIII levels in VTE patients. Importantly, further clinical trials will also be essential in order to understand the critical observation that elevated FVIII levels may also be useful in identifying a specific subset of patients who are at increased risk for developing recurrent thrombotic episodes.

Summary Points

- This chapter focuses on high plasma factor VIII levels as a biomarker for venous thromboembolism risk.
- Venous thromboembolism is a multicausal disease.
- Several genetic, acquired, and environmental factors contribute to the development of venous thromboembolism.

- Previous reports showed that increased plasma factor VIII levels are a prevalent, independent, and dose-dependent risk factor for the first episode of VTE.
- Several studies have shown that the risk of a recurrent episode is also significantly increased in patients with high FVIII levels.
- Genetics and acute phase response seem to be important players in the rising of FVIII levels.
- The molecular basis of the increased plasma FVIII levels is incompletely known.

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Carotid Artery Stenting and Outcome Predictors

31

Ali F. AbuRahma and Patrick A. Stone

Contents

Abstract

Carotid artery stenting has been advocated as an alternative therapy to carotid endarterectomy in the treatment of patients with symptomatic and asymptomatic carotid stenosis, specifically for those at high risk for surgical revascularization.

The impact of chronic renal insufficiency on the long-term clinical outcome of carotid artery stenting has not been well established. We have previously shown that although the perioperative carotid artery stenting stroke rates for normal, moderate, and severe chronic renal insufficiency were not significantly different using either serum creatinine or the glomerular filtration rate as markers, the long-term outcomes were somewhat different. The rates of freedom from major adverse events (stroke, myocardial infarction, and death) at 3 years in symptomatic patients were 81 % in patients with normal renal function versus 46 % in patients with moderate/severe chronic renal insufficiency ($p = 0.0198$). This

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_33

study also showed that the glomerular filtration rate was more sensitive in detecting late major adverse events after carotid artery stenting than serum creatinine. This study concluded that carotid artery stenting in moderate chronic renal insufficiency patients can be done with satisfactory perioperative outcomes; however the late death rate was significantly high.

Keywords
Carotid • Stenting • Outcome • Predictors

Abbreviations

CAS	Carotid artery stenting
CEA	Carotid endarterectomy
CRI	Chronic renal insufficiency
GFR	Glomerular filtration rate
MAE	Major adverse events
MDRD	Modification of diet in renal disease
MI	Myocardial infarction

Key Facts of Carotid Artery Stenting and Outcome Predictors

- Carotid artery stenting or dilatation is a procedure to open clogged arteries to the brain to prevent stroke.
- This procedure has been advocated as an alternative to the conventional open carotid surgery (carotid endarterectomy), since many feel it is a minimally invasive procedure.
- This procedure has been used with caution in patients with impaired kidney function, however it has been noted that it can be done with early satisfactory outcomes.
- The long-term effect of this procedure is somewhat guarded because the late death in these patients is higher than the normal population, therefore special consideration must be given when offering this minimally invasive procedure to patients with chronically impaired kidney function

Definitions

Carotid endarterectomy Surgical removal of plaque accumulation from the carotid artery

Creatinine Formed from the metabolism of creatine, this substance is commonly found in urine, blood, and muscle tissue and is used as an indicator of kidney function

Glomerular filtration rate A kidney function test based on the amount of ultrafiltrate formed by plasma flowing through the glomeruli of the kidney

Stroke Occlusion of the brain caused by a thrombus, embolus, or cerebrovascular hemorrhage or vasospasm resulting in ischemia of the brain tissues normally perfused by the damaged vessels

Introduction

Carotid artery stenting (CAS) has been advocated as an alternative treatment to carotid endarterectomy (CEA) for patients with symptomatic and asymptomatic carotid stenosis, especially for those who are at high risk for surgical revascularization (Mantese et al. 2010; Yadav et al. 2004; International Carotid Stenting Study Investigators 2010; EVA-3S Investigators 2004; SPACE Collaborative Group et al. 2006; Hopkins et al. 2008; Gray et al. 2006, 2007; Safian et al. 2004). The impact of chronic renal insufficiency (CRI), especially long-term clinical outcomes, on patients undergoing CAS is not well established; however a few studies have suggested that CRI is associated with an increased risk (Protack et al. 2011; Jackson et al. 2008; Saw et al. 2004; AbuRahma et al. 2014).

The prevalence of CRI in the general population has been estimated to be around 9 % (Coresh et al. 2005). CRI is associated with several advanced vascular pathologies, including coronary artery disease, cardiovascular disease, and peripheral arterial disease, which may affect operative outcome. These patients have diffused premature atherosclerosis (Porter 1985; Hellerstedt et al. 1984; Ritz et al. 1986). The annual death rates in patients with CRI are generally 20 times that of patients without CRI, and two-thirds of these deaths are secondary to cardiovascular disease (Porter 1985; Rigdon et al. 1997).

Patients with CRI are also at high risk for having a stroke, even after adjusting for traditional risk factors like diabetes mellitus. Other associated morbidities in patients with CRI, such as advanced age, hypertension, diabetes, bleeding diathesis, and malnutrition, could increase the risk of stroke in these patients. Seliger et al. concluded that patients with CRI had more severe carotid atherosclerotic disease than subjects with normal renal function (Seliger et al. 2003).

We reviewed several studies that have analyzed the best therapy for severe carotid stenosis in patients with CRI and showed increased morbidity and mortality after CEA (Rigdon et al. 1997; Plecha et al. 1993; Tarakji et al. 2006; Kretz et al. 2010; Protack et al. 2011; van Lammeren et al. 2011; Ascher et al. 2005; Sidawy et al. 2008). Some of these studies reported differences when CRI patients were separated into mild CRI (creatinine level of 1.6–2.9 mg/dL) (Rigdon et al. 1997; Kretz et al. 2010; Ascher et al. 2005; Sidawy et al. 2008) versus severe CRI (creatinine level of $\geq$3 mg/dL), with increased stroke/death only after CEA in patients with severe CRI. A few others have concluded that CAS was associated with prohibitive risks in patients with CRI and questioned its value (Protack et al. 2011; Saw et al. 2004).

We believe the variations in the results of these studies can be explained by the CRI definitions that were used. Most studies used the serum level of creatinine, while

some felt that creatinine clearance is better, and others combined both markers. Different levels of serum creatinine have been used to define the degree of CRI, and, furthermore, the methods used to estimate creatinine clearance also vary. Either the modification of diet in renal disease (MDRD) or the Cockcroft-Gault methods were used in most studies because most agree that creatinine is an insensitive marker and can remain lower than 2.0 mg/dL, despite a significant drop in the glomerular filtration rate (GFR) to as low as 15 mL/min/1.73 m^2. Therefore, the GFR is recommended by the National Kidney Foundation Kidney Disease Outcomes Quality Initiative guidelines as a better indicator for CRI.

We reviewed the only two studies (Protack et al. 2011; Saw et al. 2004) that have been published regarding the clinical outcomes of CAS in patients with CRI using the glomerular filtration rate (GFR); one was based on the Cockcroft-Gault equation (Saw et al. 2004), and the other was based on the modification of diet in renal disease (MDRD) equation (Protack et al. 2011).

Our Clinical Experience

We previously reported on a retrospective analysis of prospectively collected data of 313 CAS patients who were classified into three groups: normal (serum creatinine $<$1.5 mg/dL or GFR $\geq$60 mL/min/1.73 m^2), moderate CRI (serum creatinine 1.5–$<$3 mg/dL or GFR 30–$<$60 mL/min/1.73 m^2), and severe CRI (serum creatinine $\geq$3 or GFR $<$30 mL/min/1.73 m^2). Major adverse events ([MAE] stroke, death, and myocardial infarction) were compared for all groups.

We have previously shown that perioperative stroke rates for normal, moderate, and severe CRI were 5 %, 0 %, and 25 %, respectively, using serum creatinine ($p = 0.05$) versus 4.6 %, 3.7 %, and 11.1 %, respectively, using GFR ($p = 0.44$, Table 1). The perioperative MAE rates for symptomatic patients were 9.3 % and 0 % ($p = 0.355$) and 2 % and 5.9 % ($p = 0.223$) for asymptomatic patients for normal and moderate/severe CRI, respectively, using serum creatinine versus 8.1 % and 7.8 %, respectively, for symptomatic patients, and 2.5 % and 3 %, respectively, for asymptomatic patients using GFR (Table 2). Late MAE rates (mean follow-up of 21 months) in normal versus moderate/severe CRI patients were 8.2 % and 14 %, respectively ($p = 0.247$), using serum creatinine versus 6.6 % and 13.3 %, respectively ($p = 0.05$), using GFR. The late MAE rates for symptomatic patients in normal versus moderate/severe CRI were 8.7 % versus 27 %, respectively ($p = 0.061$), using serum creatinine and 5.7 % versus 18.8 %, respectively ($p = 0.026$), using GFR. The late death rate in normal patients was 0.55 % versus 7.6 % for patients with moderate/severe CRI ($p = 0.002$, Table 3).

Midterm Data and Kaplan-Meier Curve Analysis

Our previous study showed that freedom from stroke was not statistically significant between patients with a serum creatinine level $\geq$1.5 mg/dL versus a serum creatinine

Table 1 Perioperative complications and renal functions

Based on serum creatinine	**Normal (<1.5 mg/dL)**	**Moderate CRI (1.5–2.9 mg/dL)**	**Severe CRI (≥3 mg/dL)**	**P value**
Total number	262	47	4	
Myocardial infarction, n (%)	0	1 (2)	0	0.163
Stroke, n (%)	13 (5)	0	1 (25)	0.056
Death, n (%)	1 (0.4) 0	0	0	1
MAE (stroke, MI, death) n (%)	13 (5)	1 (2.1)	1 (25)	0.179
Based on GFR	**Normal (≥60 mL/min/1.73 m^2)**	**Moderate CRI (30–59 mL/min/1.73 m^2)**	**Severe CRI (<30 mL/min/1.73 m^2)**	**P value**
Total number	196	108	9	
Myocardial infarction, n (%)	0	1 (0.9)	0	0.374
Stroke, n (%)	9 (4.6)	4 (3.7)	1 (11.1)	0.44
Death, n (%)	0	1 (0.9)	0	0.374
MAE (stroke, MI, death), n (%)	9 (4.6)	5 (4.6)	1 (11.1)	0.666

CRI chronic renal insufficiency, *MAE* major adverse events, *GFR* glomerular filtration rate

Table 2 Perioperative complications by preoperative indication and normal renal function versus moderate/severe chronic renal insufficiency

Based on serum creatinine	**Normal (<1.5 mg/dL) ($n = 262$)**	**Moderate/Severe CRI (≥1.5 mg/dL) ($n = 51$)**	**P value**
Myocardial infarction, n (%)	0	1 (2)	0.163
Stroke, n (%)	13 (5)	1 (2)	0.481
Death, n (%)	1 (0.4)	0	1
MAE (stroke, MI, death in whole series, n (%)	13 (5)	2 (3.9)	1
MAE (188 asymptomatic patients), n (%)	3/154 (2)	2/34 (5.9)	0.223
MAE (125 symptomatic patients	10/108 (9.3)	0/17	0.355
Based on GFR	**Normal (≥60 mL/min/1.73 m^2) ($n = 196$)**	**Moderate/severe (<60 mL/min/1.73 m^2) ($n = 117$)**	**P value**
Myocardial infarction, n (%)	0	1 (0.9)	0.374
Stroke, n (%)	9 (4.6)	5 (4.3)	0.895
Death	0	1 (0.9)	0.374
MAE (stroke, MI, death) in whole series, n (%)	9 (4.6)	6 (5.1)	0.830
MAE (188 asymptomatic patients), n (%)	3/122 (2.5)	2/66 (3)	1
MAE (125 symptomatic patients), n (%)	6/74 (8.1)	4/51 (7.8)	1

CRI chronic renal insufficiency, *MAE* major adverse events, *GFR* glomerular filtration rate

Table 3 Late complications

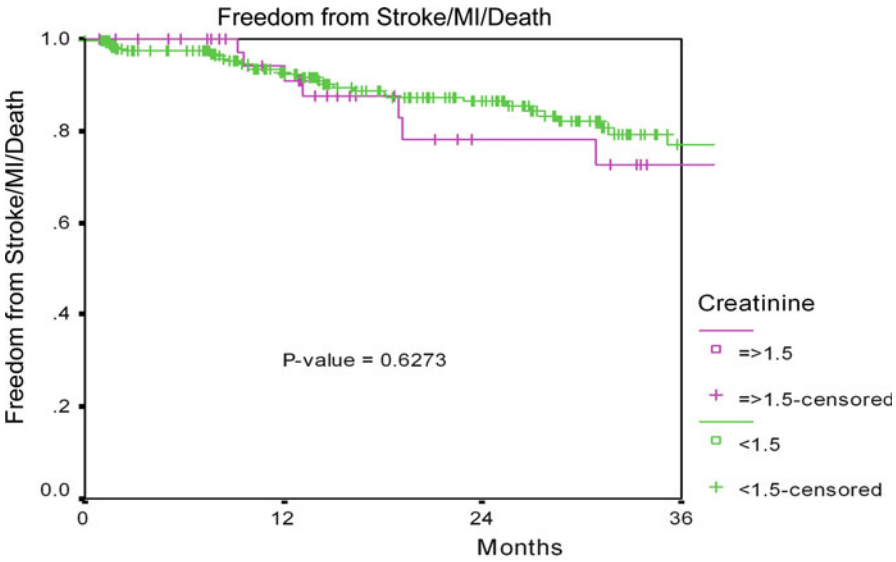
Based on serum creatinine	**Normal (<1.5 mg/dL) (*n* = 244)**	**Moderate/severe CRI (≥1.5 mg/dL) (*n* = 43)**	**P value**
Myocardial infarction, n (%)	8 (3.3)	4 (9.3)	0.0875
Stroke, n (%)	7 (2.9)	0	0.5999
Death, n (%)	7 (2.9)	2 (4.7)	0.628
MAE (stroke, MI, death) in whole series, n (%)	20 (8.2)	76 (14)	0.247
MAE (stroke, MI, death) (169 asymptomatic patients), n (%)	11/141 (7.8)	2/28 (7.1)	1
MAE (stroke, MI, death) (118 symptomatic patients), n (%)	9/103 (8.7)	4/15 (27)	0.061
Based on GFR	**Normal (≥60 mL/min/1.73 m^2) (*n* = 182)**	**Moderate/severe CRI (<60 mL/min/1.73 m^2) (*n* = 105)**	**P value**
Myocardial infarction, n (%)	6 (3.3)	6 (5.7)	0.366
Stroke, n (%)	7 (3.9)	0	0.050
Death, n (%)	1 (0.55)	8 (7.6)	0.002
MAE (stroke, MI, death) in whole series, n (%)	12 (6.6)	14 (13.3)	0.055
MAE (stroke, MI, death) (169 asymptomatic patients), n (%)	8/112 (7.1)	5/57 (8.8)	0.764
MAE (stroke, MI, death) (118 symptomatic patients), n (%)	4/70 (5.7)	9/48 (18.8)	0.026

level <1.5 mg/dL. At 3 years, the rates of freedom from stroke were 85 % for patients with normal renal function versus 97 % for patients with a serum creatinine ≥1.5 mg/dL ($p = 0.1811$). Similarly, at 3 years, the rate of freedom from stroke for patients with a GFR ≥60 mL/min/1.73 m^2 was 84 % versus 93 % for patients with a GFR <60 mL/min/1.73 m^2 ($p = 0.3127$). Figures 1 and 2 show the rates of freedom from MAE (stroke, myocardial infarction [MI], and/or death) at 3 years: 77 % for patients with a serum creatinine <1.5 mg/dL versus 73 % for patients with a serum creatinine ≥1.5 ($p = 0.6273$), in contrast to 81 % for patients with a GFR ≥60 mL/min/1.73 m^2 versus 68 % for patients with a GFR <60 mL/min/1.73 m^2 ($p = 0.1015$). Figures 3 and 4 show the freedom from stroke, MI, and/or death based on the indications for CAS, creatinine levels, and GFR. At 3 years, the rate of freedom from stroke, MI, and death was 73 % for symptomatic patients with a serum creatinine <1.5 mg/dL versus 53 % for symptomatic patients with a serum creatinine ≥1.5 mg/dL and 80 % for asymptomatic patients with a creatinine <1.5 mg/dL versus 81 % for asymptomatic patients with creatinine ≥1.5 mg/dL ($p = 0.1544$). However, at 3 years, symptomatic patients with a GFR of <60 mL/min/1.73 m^2 had a freedom from stroke, MI, and/or death rate of 46 % versus 81 % for symptomatic patients with a GFR of ≥60 mL/min/1.73 m^2 ($p = 0.0198$, Fig. 4). The rates for asymptomatic patients were the same (81 %) for patients with a GFR of <60 mL/min/1.73 m^2 and ≥60 mL/min/1.73 m^2.

A multivariate Cox regression analysis showed that a GFR of <60 mL/min/1.73 m^2 had an odds ratio of 1.6 ($p = 0.222$) of having a MAE after CAS (Table 4).

Comments

We reviewed several prospective randomized trials that have been conducted to analyze the efficacy of CAS for both high-risk and low-risk patients, with mixed conclusions. These trials included Carotid Revascularization with Endarterectomy or Stent Trial (CREST), which compared CAS and CEA in patients with asymptomatic and symptomatic carotid stenosis (Mantese et al. 2010); the Stent-Protected Angioplasty versus Carotid Endarterectomy trial (SPACE) (SPACE Collaborative Group et al. 2006); the Endarterectomy versus Angioplasty in Patients with Symptomatic Severe Carotid Stenosis (EVA-3S) trial (EVA-3S Investigators 2004); the International Carotid Stenting Study (ICSS) (International Carotid Stenting Study Investigators 2010), which is a multinational prospective randomized trial comparing CEA or CAS for symptomatic patients; and the Stenting and Angioplasty with Protection in Patients at High Risk for Endarterectomy trial (SAPPHIRE) (Yadav et al. 2004). We also reviewed several carotid registries that have reported on the outcome of CAS in both asymptomatic and symptomatic patients, such as the Boston Scientific EPI: A Carotid Stenting Trial for High-Risk Surgical Patients trial (BEACH) (White et al. 2006), Carotid Acculink/Accunet Post Approval Trial to Uncover Unanticipated or Rare Events trial (CAPTURE) (Gray et al. 2007), Acculink for Revascularization of Carotids in High-Risk patients trial (ARCHeR) (Gray et al. 2006), and



At Risk	<1.5	244	152	94	38
	≥1.5	43	30	14	8
Percent	<1.5	100%	93%	86%	77%
	≥1.5	100%	91%	78%	73%
SE	<1.5		1.9%	2.7%	4.2%
	≥1.5		5.0%	8.1%	9.3%

Fig. 1 Freedom from stroke/MI/death based on serum creatinine levels

the Carotid Artery Revascularization using the Boston Scientific FilterWire EX/EZ and the EndoTex NexStent trial (CABERNET) (Hopkins et al. 2008).

Unfortunately, patients with CRI have been excluded from most of the clinical trials that have analyzed the outcomes of CAS. In addition, the use of contrast during CAS may also cause contrast-induced nephropathy and exaggerate CRI. Furthermore, dialysis patients are prone to bleeding and may necessitate close observation if dual antiplatelets are used during and after stenting. Jackson et al. (2008) evaluated perioperative complications (stroke MI, death, and femoral artery pseudoaneurysms) after 215 CAS procedures. This study showed that the perioperative minor and major stroke rates were 2.8 % and 0.5 %, respectively, and that CRI was a predictor of perioperative complications, including stroke. Patients with normal renal function had a 13 % complication rate and a 0.6 % stroke rate versus a 37 % complication rate and an 11.1 % stroke rate in patients with a serum creatinine >1.3 mg/dL ($p = 0.003$ and $p = 0.0001$) (Jackson et al. 2008)

We reviewed a report by Saw et al. (2004) on the clinical outcome of patients who had CAS procedures at the Cleveland Clinic during a 5-year period. The Cockcroft-Gault equation was used to calculate creatinine clearance. Patients were classified according to the presence or absence of CRI based on their GFR (<60 mL/min/1.73 m^2). Patients were divided into four groups based on their creatinine clearance

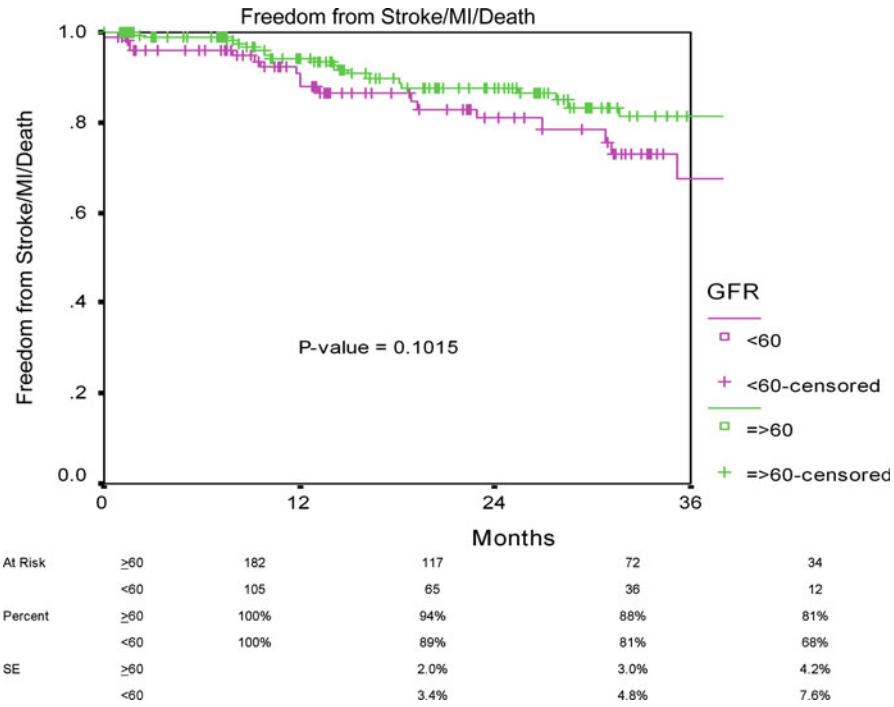
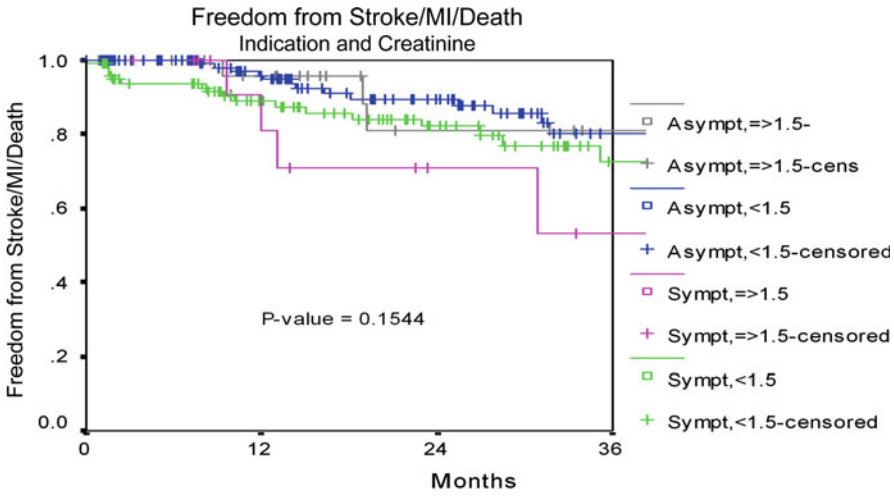


Fig. 2 Freedom from stroke/MI/death based on GFR levels

quartiles. Five hundred eighty-one patients who underwent CAS had creatinine clearance data. This study showed that patients with CRI had a higher combined MAE rate at 7 days (6.8 % versus 2.7 %, $p = 0.023$) and at 6 months (14.7 % versus 5.6 %, $p < 0.001$), compared to patients without CRI. CRI was also associated with a higher stroke rate (4.2 % versus 0.8 %, $p = 0.009$) and death rate (8.4 % versus 3.4 %, $p = 0.015$) at 6 months compared to patients with no CRI. The Kaplan-Meier MAE-free survival at 6 months was significantly higher in patients without CRI than those with CRI ($p < 0.001$). When clinical outcomes were compared using the creatinine clearance quartiles, the highest creatinine clearance quartile (fourth quartile) was associated with a lower combined MAE rate at both 7 days (1.4 % versus 6.2 %, $p = 0.049$) and at 6 months (4.1 % vs. 14.5 %, $p = 0.004$), compared to the lowest creatinine clearance quartile. A Cox regression analysis revealed that a previous MI, CRI, diabetes mellitus, age, and baseline hemoglobin were univariate predictors of 6-month combined MAE rates. A multivariate analysis showed that only diabetes and CRI were independent predictors of 6-month combined MAE rates.

Similar observations were noted by Protack et al. (2011), who evaluated the effect of CRI on outcomes after all modalities of carotid revascularization. Patients were classified based on GFR (MDRD equation). This study showed that the 30-day mortality and morbidity rates were 1.1 % and 16.9 %, respectively. Moderate CRI



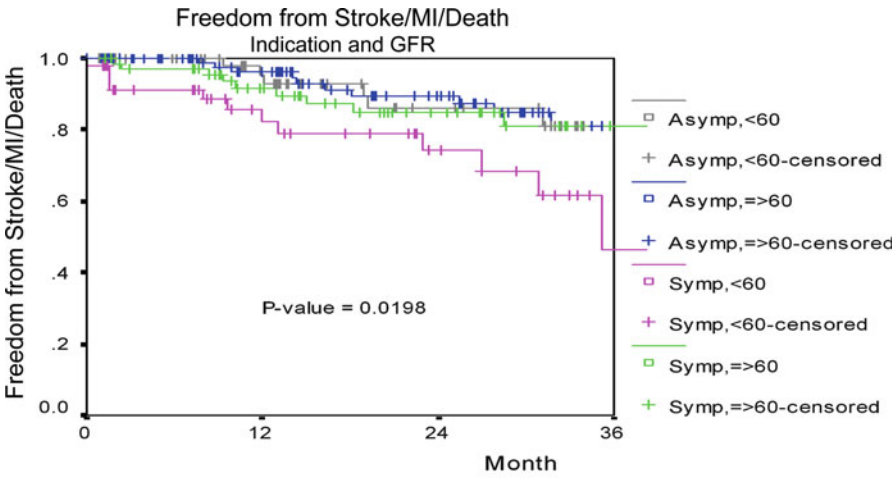
At Risk	Sympt, < 1.5	103	64	38	16
	Sympt, ≥ 1.5	15	9	4	2
	Asympt, < 1.5	141	88	56	22
	Asympt, ≥ 1.5	28	21	10	6
Percent	Sympt, < 1.5	100%	89%	82%	73%
	Sympt, ≥ 1.5	100%	81%	71%	53%
	Asympt, <1.5	100%	96%	90%	80%
	Asympt, ≥ 1.5	100%	96%	81%	81%

Fig. 3 Freedom from stroke/MI/death by CAS indication and creatinine

was present in 28 % of patients and 6 % had severe CRI. The 30-day stroke rates were 3 %, 2.7 %, and 5.5 % for normal renal function, moderate CRI, and severe CRI, respectively ($p = 0.54$). The 30-day mortality rates were 0.7 % for the normal renal function group, 1.2 % for the moderate CRI group, and 5.5 % for the severe CRI group ($p = 0.005$). CAS patients with severe CRI showed significantly lower rates of freedom from stroke. Overall, patients with moderate CRI had similar outcomes; however patients with severe CRI had significantly higher 30-day death rates after carotid revascularization (Protack et al. 2011).

Potential Applications to Prognosis and Other Diseases or Conditions

The effect of chronic kidney disease (chronic renal insufficiency) on long-term clinical outcomes of certain procedures has been controversial. Its effect on both carotid endarterectomy and carotid stenting has been studied, with mixed results. Its effect on early and long-term durability of carotid stenting for the prevention of stroke has only been studied in a few series. Most of these studies analyzed the impact of serum creatinine and its level on the outcome of carotid intervention,



At Risk	Sympt, ≥60	70	47	28	15
	Sympt, <60	48	26	14	3
	Asympt, ≥60	112	70	44	19
	Asympt, <60	57	39	22	10
Percent	Sympt, ≥60	100%	92%	85%	81%
	Sympt, <60	100%	82%	74%	46%
	Asympt, ≥60	100%	96%	90%	81%
	Asympt, <60	100%	95%	86%	81%

Fig. 4 Freedom from stroke/MI/death by CAS indication and GFR

Table 4 Cox regression analysis of early or late stroke, death, or myocardial infarction after carotid artery stenting

Effect	Hazard ratio	95 % Wald confidence limits	P value
Univariate			
Creatinine ≥1.5 ml/dL	1.2	0.5–2.7	0.674
GFR < 60 ml	1.6	0.9–3.1	0.127
Diabetes mellitus	1.1	0.6–2.0	0.832
Hypertension	0.7	0.3–1.7	0.456
Coronary artery disease	1.5	0.7–3.5	0.306
Congestive heart failure	1.5	0.7–3.1	0.259
Smoker	0.7	0.4–1.3	0.216
Hypercholesterolemia	1	0.5–1.9	0.990
Asymptomatic	0.5	0.3–1	0.035
Prior CEA	0.3	0.1–0.5	0.0002
Multivariate			
Creatinine ≥1.5 mg/dL	0.95	0.4–2.5	0.918
GFR <60 ml	1.6	0.8–3.3	0.222
Prior CEA	0.3	0.15–0.6	0.0006
Asymptomatic	0.6	0.3–1.1	0.095

however very few have analyzed the effect of the glomerular filtration rate (GFR) utilizing the modification of diet in renal disease (MDRD) method as a marker to determine the efficacy of this procedure, both early and late. This study emphasized the significance of this biomarker, specifically the GFR using the MDRD, and its impact on offering carotid stenting as an alternative to carotid endarterectomy in patients with chronic renal disease. Based on this review, we also concluded that although carotid stenting can be offered as an alternative to carotid endarterectomy with satisfactory results, the late death is significantly higher than desired; therefore, caution should be exercised in these patients using this biomarker.

Summary Points

- Chronic renal insufficiency had no effect on perioperative outcomes after carotid artery stenting, regardless of whether serum creatinine or glomerular filtration rate was used.
- Glomerular filtration rate is more sensitive in predicting late major adverse events, especially in symptomatic patients.
- Carotid artery stenting can be performed in patients with moderate and severe chronic renal insufficiency with satisfactory perioperative outcomes.
- The late death and late major adverse event rates are significantly higher in patients with moderate and severe chronic renal insufficiency, especially in symptomatic patients.
- Carotid artery stenting should be guarded in these patients.

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Part IV

Molecular, Cellular, and Histological Variables

RhoA/Rho-Associated Kinase as Marker of Cardiovascular Health

32

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_17

Abstract

Rho-associated coiled-coil kinase (ROCK) is a widely expressed intracellular signaling molecule. ROCK is the best-described downstream regulator of Rho and regulates numerous cellular functions such as division, migration, growth, and death. ROCK activity has been implicated in the development of various forms of cardiovascular disease including stroke, coronary artery disease, hypertension, and several others. While much effort has been devoted to the study of ROCK inhibitors and their potential role in the treatment of cardiovascular disease, a growing body of evidence suggests that HMG-CoA reductase inhibitors (statins) indirectly inhibit ROCK by preventing the necessary preceding step of Rho tethering to the plasma membrane. In this way, statins are believed to exert beneficial cholesterol-independent or "pleiotropic" effects. In the following chapter, we will discuss the molecular structure of ROCK, the regulation of the Rho/ROCK pathway, the role of Rho/ROCK in numerous forms of cardiovascular disease, and the available evidence implicating ROCK as a pharmaceutical target.

Keywords

Rho-associated coiled-coil kinase (ROCK) • HMG-CoA reductase inhibitors (statins) • Cardiovascular disease • Stroke • Atherosclerosis • Pulmonary arterial hypertension • Fasudil

Abbreviations

ACS	Acute coronary syndrome
AGE	Advanced glycation end products
ApoE	Apolipoprotein-E
BMI	Body mass index
CAD	Coronary artery disease
DNA	Deoxyribonucleic acid
EKG	Electrocardiogram
eNOS	Endothelial nitric oxide synthase
ERM	Ezrin-radixin-moesin
FMD	Flow-mediated dilatation
FPP	Farnesyl pyrophosphate
GGPP	Geranylgeranyl pyrophosphate
GTP	Guanosine-5′-triphosphate

HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
hsCRP	High-sensitivity C-reactive protein
ICAM	Intercellular adhesion molecule
IF	Intermediate filaments
IFN	Interferon
IL	Interleukin
IRS	Insulin receptor substrate
JNK	c-Jun NH(2)-terminal kinase
KD	Knockdown
LDL	Low-density lipoprotein
LIMK	(Lin11, Isl-1, and Mec-3) domain kinase
L-NAME	N^{ω}-Nitro-L-arginine methyl ester
LPA	Lysophosphatidic acid
MBS	Myosin-binding subunit
MCA	Middle cerebral artery
MCP	Monocyte chemoattractant protein
MLC	Myosin light chain
MLCP	Myosin light chain phosphatase
NF-κB	Nuclear factor κB
NO	Nitric oxide
NOS	Nitric oxide synthase
NSTEMI	Non-ST segment elevation myocardial infarction
NT-pro-BNP	N-terminal prohormone of brain natriuretic peptide
PAD	Peripheral arterial disease
PAH	Pulmonary arterial hypertension
PAI	Plasminogen activator inhibitor
PCI	Percutaneous coronary intervention
PDGF	Platelet-derived growth factor
PH	Pleckstrin homology
PIP	Phosphatidylinositol (3,4,5)-trisphosphate
PIP5K	Phosphatidylinositol 4-phosphate 5-kinase
PKC	Protein kinase C
PTEN	Phosphatase and tensin homologue
RBD	Rho-binding domain
ROCK	Rho-associated coiled-coil kinase
ROS	Reactive oxygen species
S1P	Sphingosine-1-phosphate
SNP	Single-nucleotide polymorphism
STEMI	ST segment elevation myocardial infarction
TCR	T-cell receptor
TGF	Transforming growth factor
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
VSMCs	Vascular smooth muscle cells
WT	Wild type

Definitions

Actomyosin Bundles A complex of actin and myosin which forms the basic subunit of contractile protein filaments. Actomyosin bundles are responsible for the contraction of muscle cells but are also important for cellular migration and intracellular trafficking in numerous cell types.

Apoptosis A process of programmed cell death whereby cells undergo a series of biochemical events leading to fragmentation of the cell and eventual consumption by phagocytes. Apoptosis is a generally advantageous method of cell death, in contrast with necrosis, as fragmentation occurs in a controlled fashion which prevents damage to neighboring cells. Apoptosis is a naturally occurring process in organism growth and development.

Fasudil A potent pharmacologic inhibitor of ROCK. Fasudil nonselectively inhibits both ROCK isoforms which is a major limitation to its clinical use.

Hypertension A chronic condition where the systemic arterial blood pressure is elevated above normal levels (above 140/90 mmHg). Hypertension is a risk factor for all known forms of cardiovascular disease.

Metabolic Syndrome A common medical disorder characterized by 3 out of 5 of the following conditions: hypertension, elevated fasting plasma glucose, abdominal obesity, high triglycerides, and low HDL cholesterol. Metabolic syndrome is a major risk factor for the development of diabetes mellitus, coronary artery disease, and numerous other medical problems.

Myocardial Hypertrophy A condition where the heart muscle has become thickened often due to pressure overload on the heart. Hypertrophy may impair the filling of the heart and may cause the pumping chambers of the heart to get smaller.

Nitric Oxide (NO) A powerful vasodilatory signaling molecule. In the vascular endothelium, NO is produced by endothelial nitric oxide synthase (eNOS). It then diffuses out of the endothelial cell and into neighboring smooth muscle cells where it binds to and activates guanylate cyclase, producing cGMP which results in reuptake of calcium into the sarcoplasmic reticulum and relaxation of the smooth muscle cell.

Pleckstrin Homology (PH) Motif A protein domain which allows binding of the peptide to phosphatidylinositol lipids in biological membranes. PH domains are important parts of heterotrimeric G proteins as well as proteins that bind to the plasma membrane.

Pleiotropic Effects of Statins Beneficial effects of statins that are independent of their cholesterol-lowering effects. One explanation for these effects involves a decrease in the bioavailability of GGPP which prevents Rho activation and thereby

inhibits ROCK activity. There are likely several other mechanistic possibilities for statins' pleiotropic effects. This is an area of active research and great clinical interest.

Pulmonary Arterial Hypertension A chronic progressive medical condition where the blood pressure in the pulmonary vasculature is elevated above normal levels. This increases the workload on the right side of the heart. In severe cases, pulmonary hypertension can result in right heart failure and death.

Introduction

Rho-associated coiled-coil kinase (ROCK) is the best-described downstream effector of the small GTPase RhoA and is a main regulator of actin cytoskeletal organization. ROCK regulates numerous cellular functions such as proliferation, apoptosis, adhesion, contraction, and migration. Studies in humans have suggested a role for ROCK in various cardiovascular disease states such as atherosclerosis, hypertension, stroke, pulmonary arterial hypertension, and heart failure. Numerous in vitro and animal studies exploring the physiological roles of ROCK have exploited genetic manipulation of ROCK as well as pharmacologic inhibition. Such studies have demonstrated the role of ROCK inhibition in reducing endothelial dysfunction, inflammation, negative remodeling of the vasculature or myocardium, and ischemia-reperfusion injury. This chapter will provide an overview of the current scientific understanding of the ROCK molecule and its physiologic implications. The reader is directed to the references (specifically those listed in the tables) for details about particular experimental models and results (Tables 1, 2, and 3).

ROCK Structure and Function

The Rho-GTPase Family

The Rho-GTPase family of proteins function as signal transducers between receptors on the cell surface and intracellular signaling pathways. The family is comprised of three members: Rho (including RhoA, RhoB, and RhoC), Rac, and CDC42, and activity is enhanced through GTP binding (Leung et al. 1996). The Rho-GTPases are critical regulators of the cell cycle, proliferation, and apoptosis, and have been shown to regulate several movement-related processes such as chemotaxis and contraction. The family of serine/threonine protein kinases, known as ROCKs, are 160 kDa downstream effectors of Rho (Riento and Ridley 2003). ROCKs are integral to regulating the organization and dynamics of the intracellular actin cytoskeleton. The family contains two known isoforms ROCK1 and ROCK2, which share 65% amino acid sequence homology and 92% homology within their kinase domains (Nakagawa et al. 1996). Both ROCK isoforms are widely expressed.

Table 1 Rho-associated coiled-coil kinase targets relevant to cardiovascular disease

Downstream target	Effect of ROCK on target function	References
Desmin	Reduces intermediate filament assembly	(Inada et al. 1998)
EF1α	Reduces F-actin binding	(Izawa et al. 2000)
eNOS	Decreases phosphorylation, inactivates NO synthetic activity	(Ming et al. 2002; Sugimoto et al. 2007)
ERM proteins	Increases F-actin assembly, regulates cytoskeletal remodeling, regulates head-to-tail associations	(Matsui et al. 1998; Oshiro et al. 1998; Haas et al. 2007; Chen et al. 2011)
IRS-1	Reduces (phosphorylation of Ser307) or increases (Ser632/635) insulin signaling	(Farah et al. 1998; Begum et al. 2002; Furukawa et al. 2005; Lim et al. 2007)
JIP-3	Inhibits JNK signaling	(Ongusaha et al. 2008)
LIM Kinases	Increases kinase activity	(Ohashi et al. 2000; Sumi et al. 2001)
MLC	Increases actin-myosin stress fiber assembly	(Totsukawa et al. 2000)
MYPT1	Reduces MLCP activity	(Kimura et al. 1996)
PTEN	Increases phosphatase activity	(Li et al. 2005)
RhoE	Increases RhoE stability	(Hall 1994)
Troponin	Sensitizes cardiomyocytes to calcium	(Vahebi et al. 2005)
Vimentin	Increases intermediate filament disassembly	(Goto et al. 1998; Nakamura et al. 2000)
ZIPK	Increases ZIPK activity	(Riento et al. 2005)

This table lists ROCK substrate molecules relevant to cardiovascular disease. As shown, many ROCK targets are involved in actin cytoskeleton regulation. *EF1α* elongation factor 1α, *eNOS* endothelial nitric oxide synthase, *ERM* ezrin-radixin-moesin, *IRS-1* insulin receptor substrate 1, *JIP-3* JNK-interacting protein 3, *LIMK 1/2* (Lin11, Isl-1, and Mec-3) domain kinase 1 and 2, *MLC* myosin light chain, *MYPT1* myosin phosphatase target subunit 1, *PTEN* phosphatase and tensin homologue, *ZIPK* zipper-interacting protein kinase

Table 2 Key facts of atherosclerosis

Atherosclerosis is a disease process where fatty plaque builds up in the arteries
Atherosclerosis leads to compromised blood flow and is the primary pathophysiological process in coronary artery disease, peripheral arterial disease, and some forms of stroke
In acute coronary syndrome, blood flow to the heart muscle is seriously reduced causing life-threatening tissue ischemia
In severe cases, acute coronary syndrome may lead to myocardial infarction or death
Endothelial dysfunction precedes atherosclerosis, and endothelial function can be measured by flow-mediated dilatation
ROCK activity has been implicated in the development of endothelial dysfunction and atherosclerosis in animal models, but relevant human studies remain few

This table lists key facts about atherosclerosis including pathophysiology, clinical implications, and the relationship with endothelial dysfunction

Table 3 Key facts of stroke

Stroke is a leading cause of death worldwide
Stroke occurs when blood flow to a portion of the brain is compromised
There are several types of stroke:
Hemorrhagic stroke occurs when a blood vessel in the brain bursts
Ischemic stroke occurs when a blood vessel in the brain becomes blocked either from atherosclerotic plaque or from embolic material (thrombus, calcium, etc.) from the heart
While stroke causes injury to the brain, further damage is caused by the inflammation that follows stroke
ROCK activity has been implicated both in the development of atherosclerosis which leads to stroke and in the inflammation that follows
ROCK inhibition may be a promising therapeutic target in the treatment of stroke

This table lists key facts about stroke including pathophysiology, clinical implications, and the role of ROCK in ischemic injury to the brain

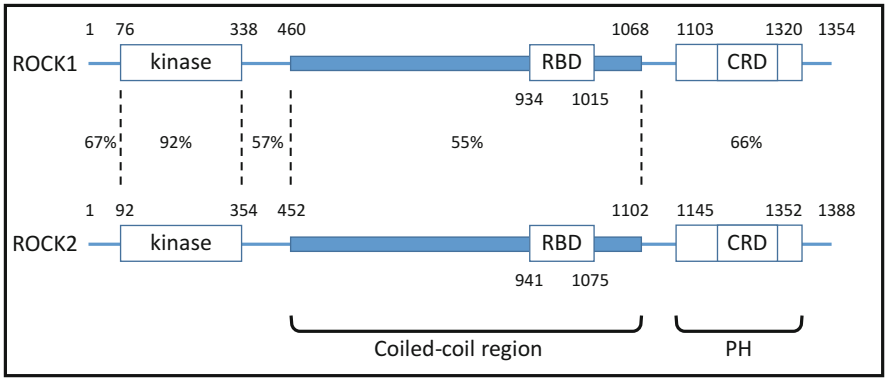

Fig. 1 The homology between ROCK1 and ROCK2 isoforms. Both isoforms consist of an N-terminal kinase domain, an RBD within a coiled-coil forming region, and a CRD within the PH. ROCK isoforms share 65 % overall homology and 92 % homology within their kinase domains. *CRD* carboxy-terminal cysteine-rich domain, *PH* pleckstrin homology, *RBD* Rho-binding domain, *ROCK* Rho-associated coiled-coil kinase

However, despite their homology, ROCK1 and ROCK2 have widely differing functions.

ROCK Structure and Regulation

Both ROCK isoforms share a highly conserved amino-terminal kinase domain, a central coiled-coil region which forms the Rho-binding domain (RBD), and a cysteine-rich domain at the carboxy-terminus within a pleckstrin homology (PH) motif (Schofield and Bernard 2013; Sawada and Liao 2014). The comparative structures of ROCK1 and ROCK2 are shown in Fig. 1. The carboxy-terminus of

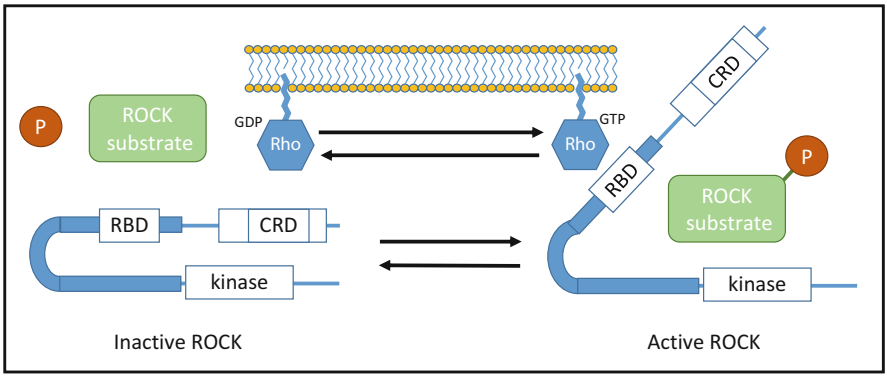

Fig. 2 The mechanism of ROCK activation by Rho-GTP. While inactive, the PH and RBD domains bind to the N-terminus of the enzyme to form an auto-inhibitory loop. Binding of active Rho-GTP to the RBD causes a conformational change, opening the enzyme and exposing the kinase domain. *CRD* carboxy-terminal cysteine-rich domain, *PH* pleckstrin homology, *RBD* Rho-binding domain, *ROCK* Rho-associated coiled-coil kinase

the molecule serves as a tail-like autoregulatory subunit which sterically inhibits the activity of the amino-terminal kinase domain under basal conditions through intermolecular association (Amano et al. 1999) ("closed conformation"). ROCK classically is activated through the binding of the Rho-GTP complex to the RBD. As shown in Fig. 2, when GTP-bound Rho interacts with the RBD, the ROCK molecule undergoes a conformational change resulting in repression of the RBD and PH domains from the kinase domain ("open conformation"), allowing binding and phosphorylation of the substrate.

ROCK activation also occurs through several Rho-A-independent mechanisms (Schofield and Bernard 2013; Sawada and Liao 2014). During the process of apoptosis, cleavage of the carboxy-terminal tail of ROCK1 by caspase 3 results in constitutive activity of ROCK1, deregulated phosphorylation of downstream targets including myosin light chain, membrane blebbing, and fragmentation of DNA (Coleman et al. 2001; Sebbagh et al. 2001). ROCK activity can also be induced by binding of arachidonic acid to the PH domain which induces a similar "open conformation" to the action of RhoA; however, this mechanism appears to be independent of Rho (Feng et al. 1999). Gem and Rad (two other small GTP-binding proteins) appear to negatively regulate ROCK activity in specific cell types such as fibroblasts through an unclear mechanism (Ward et al. 2002). RhoA-independent activation of ROCK can also occur by oligomer transphosphorylation of the amino terminus (Chen et al. 2002; Turner et al. 2002). Finally, ROCK1 participates in autoregulation through the phosphorylation of RhoE which binds to the kinase domain of ROCK1 (not the RBD) leading to a conformational change that prevents RhoA binding to the RBD and inhibits ROCK1 activity (Riento et al. 2003). Taken together, the current evidence suggests that both Rho-dependent and Rho-independent mechanisms can activate either ROCK1 or ROCK2 resulting in situation-specific cellular effects.

Commonly used inhibitors of ROCK activity include fasudil which is widely studied, and the more novel Y-27632. Both of these are discussed below.

Expression of ROCK1 and ROCK2

ROCK1 and ROCK2 are encoded on chromosomes 18q11 and 2p24, respectively, and their genes contain 33 exons (Takahashi et al. 1998). ROCKs are constitutively expressed in all embryonic and adult tissues. However, the relative expression of each isoform varies by tissue type. ROCK1 is widely expressed in organs such as the kidney, liver, spleen, lung, and testis (Schofield and Bernard 2013). Conversely, ROCK2 is more narrowly expressed, mostly in the vasculature, brain, and heart (Nakagawa et al. 1996). Within the cell, the distribution of ROCK1 and ROCK2 may differ. ROCK2 is primarily cytosolic where it associates with cytoskeleton actin fibers (Katoh et al. 2001; Chen et al. 2002). The precise location(s) of intracellular ROCK1 expression however is less well defined (Schofield and Bernard 2013).

Substrates

ROCK1 and ROCK2 regulate a variety of cellular processes such as growth, movement, and apoptosis though the phosphorylation of targets involved in actin/myosin bundle assembly, intermediate filaments, and a variety of other proteins (Schofield and Bernard 2013; Sawada and Liao 2014). This regulation occurs both through direct regulation of the cytoskeleton as well as through intermediate molecules. The phosphorylation of myosin light chain (MLC), a key promoter of cellular contraction, depends on the balance of phosphorylation and dephosphorylation by MLC kinase and MLC phosphatase, respectively, and is tightly regulated through the action of ROCK.

ROCK2 can phosphorylate MLC directly on Ser19 (Amano et al. 1996), but the contribution of ROCK to overall MLC phosphorylation is minimal through this pathway. Rather, ROCK primarily drives MLC phosphorylation through phosphorylation of the myosin-binding subunit (MBS) on MLC phosphatase which inhibits its activity (Feng et al. 1999). Further, exerts an indirect effect on cytoskeletal rearrangement through intermediate pathways. Both ROCK1 and ROCK2 phosphorylate and activate the serine/threonine kinases LIMK1 and LIMK2 which in turn phosphorylate and inhibit the actin depolymerizing factor, cofilin (Katoh et al. 2001). Through the inhibition of cofilin, ROCKs exert a net positive effect on actin polymerization. Additionally, ROCK2 phosphorylates the subunits of the Ezrin/Radixin/Moesin (ERM) family of proteins which regulate cross-linking of F-actin. This phosphorylation increases intermolecular binding among the subunits, stabilizing the complex and allowing translocation to the plasma membrane and cytoskeletal rearrangement (Matsui et al. 1998). The action of ROCK on the ERM pathway is likely critical in the development of atherosclerotic plaques (Rekhter et al. 2007).

In addition to its effects on actomyosin bundles, ROCK influences the cytoskeleton through regulation of intermediate filaments (IFs) (Schofield and Bernard

2013). ROCKs phosphorylate several IF proteins such as desmin and vimentin. Desmin is an important component of sarcomeres in all muscle types and vimentin regulates the epithelial-mesenchymal transition. Phosphorylation of either protein by ROCK inhibits IF assembly (Inada et al. 1998; Lee et al. 2006). Through balanced regulation of actomyosin bundle formation and intermediate filaments, ROCKs influence a wide variety of cellular processes.

Physiologic Functions

ROCKs are critical mediators of numerous physiologic functions. As mentioned above, ROCKs regulate cellular growth, metabolism, migration, and apoptosis through control of actin cytoskeletal assembly and cell contraction (Noma et al. 2006). ROCKs importantly phosphorylate and inhibit myosin light chain phosphatase (MLCP), increasing MLC phosphorylation which promotes cellular contraction through increased formation of actin/myosin bundles. Further, ROCKs regulate cell migration and polarity by controlling tight and adherens junctions through cytoskeletal contractions. In this way, ROCKs regulate macrophage phagocytic activity and endothelial cell permeability (Wojciak-Stothard et al. 2001; Wojciak-Stothard and Ridley 2002).

ROCKs also perform physiologic functions independent of the actin cytoskeleton. ROCKs play a vital role in maintaining vascular homeostasis and in coordinating function between endothelial cells and vascular smooth muscle cells (VSMCs) (Sawada and Liao 2014). While the complete mechanism is incompletely understood, there is growing evidence that activation of the NO/cGK pathway inhibits RhoA/ROCK signaling leading to antagonistic effects on the VSMCs. In a rat model, the NO-donor sodium nitroprusside inhibits phenylephrine-induced RhoA translocation from the cytosol to the endothelial membrane in the aorta, an action that deactivates ROCK (Sauzeau et al. 2000). Further, NO-induced vasodilation of the aorta has been shown to be at least partly mediated by inhibition of RhoA/ROCK signaling in a rat model (Chitaley and Webb 2002). Similarly, nitric oxide synthase (NOS) inhibition has also been shown to increase activation of ROCK (Carter et al. 2002) and promote vascular inflammation and remodeling (Kataoka et al. 2002). Given the well-known role of NO in decreasing vascular inflammation and inducing vessel relaxation, this evidence suggests that the antagonistic actions of NO and ROCK in the vasculature are a likely cause for the development of multiple forms of cardiovascular disease such as hypertension, atherosclerosis, and fibrosis.

ROCK and Cardiovascular Disease

Atherosclerosis and Coronary Artery Disease

Atherosclerosis is a common form of cardiovascular disease involving lipid deposition in a vessel wall followed by inflammation and thrombus formation. Activation of the Rho/ROCK pathway is a critical step for many phases of atherogenesis in

various animal models (Sawada and Liao 2014). In an LDL receptor-deficient mouse model of atherosclerosis, pharmacologic ROCK inhibition with Y-27632 significantly reduced atherosclerotic lesion formation (Mallat et al. 2003). Similarly, in a mouse model of early atherosclerosis due to apolipoprotein-E (ApoE) deficiency and carotid artery ligation, ROCK inhibition with Y-27632 prevented lesion formation and inhibited phosphorylation of ROCK targets such as ezrin-radixin-moesin (ERM) proteins in the endothelium and macrophages (Rekhter et al. 2007). Highlighting the role of ROCK in recruiting macrophages to atherosclerotic lesions, transplant of bone marrow from ROCK2 (+/− or −/−) mice into LDL receptor knockout mice is sufficient to reduce high-fat diet-induced lipid accumulation in the aorta, decrease atherosclerotic lesion formation in the subaortic sinus, and decrease foam cell formation (Zhou et al. 2012). Additionally, chronic ROCK inhibition with fasudil induces regression of IL-1ß-induced atherosclerotic coronary lesions in a porcine model (Shimokawa et al. 2001).

Evidence also supports the role of ROCK in lesion formation after vascular injury. Bone marrow-specific knockdown of ROCK1 (but not ROCK2) protects against neointimal formation and inflammatory cell infiltration in a mouse model of vascular injury, and WT to ROCK1 KD bone marrow transplant is sufficient to restore neointimal formation and inflammatory cell infiltration in these mice (Noma et al. 2008). In an L-NAME-induced rat model of vascular inflammation and atherosclerosis, treatment with Y-27632 prevented early inflammation and coronary atherosclerosis and prevented upregulation of monocyte chemoattractant protein-1 (MCP-1) and transforming growth factor-β1 (TGF-β1), two important inflammatory cytokines (Kataoka et al. 2002). In ROCK1 haploinsufficient mice, pharmacologic induction of cardiac perivascular fibrosis with angiotensin II was reduced compared with WT (Rikitake et al. 2005b). Further, pharmacologic inhibition of ROCK has been shown to decrease neointimal hyperplasia in balloon-injured carotid arteries in a rat model (Sawada et al. 2000), suggesting a pivotal role for ROCK as a promoter of vascular proliferative disorders such as in-stent restenosis after PCI.

While the wealth of evidence implicating ROCK in atherosclerosis is far less in humans than in relevant disease models, the existing evidence corroborates the data from animal studies. In patients with acute coronary syndrome (ACS), ROCK activity was increased in patients with STEMI, NSTEMI, and unstable angina (Dong et al. 2013b). Further, patients with an elevated NT-pro-BNP and high ROCK activity had a fivefold risk of a cardiovascular event compared to those with normal BNP and ROCK activity (Dong et al. 2013b). In a separate study, leukocyte ROCK activity was an independent prognostic indicator following PCI in human patients with CAD (Liu et al. 2014). In men without known cardiovascular disease, ROCK activity correlates negatively with endothelial function measured by flow-mediated dilatation (FMD), systolic blood pressure, LDL, BMI, and Framingham risk score (Soga et al. 2011). In this study, FMD was also an independent predictor of ROCK activity. In a similar study in patients who smoke, ROCK activity was elevated, and FMD was impaired in smokers compared with non-smokers, and ROCK activity again correlated negatively with FMD in the smoking cohort (Hidaka et al. 2010). Given the known negative cardiovascular effects of

smoking, these data highlight the role of ROCK in the development of cardiovascular disease.

The role of ROCK in humans with angina however has been less straightforward. In patients with stable angina, treatment with fasudil 80 mg three times daily improved exercise tolerance and ischemic threshold (as measured by ST segment depression on EKG), but no significant difference was found in time to angina, nitroglycerin use, frequency of angina, or Canadian Cardiovascular Society class (Vicari et al. 2005). Interestingly, while fasudil improved endothelial function (as measured by endothelium-dependent vasodilation) and reduced ROCK overactivity in human patients with CAD (Nohria et al. 2006), no change in ROCK activity was found in normal patients treated with fasudil. In fact, endothelial function in these healthy patients actually tended to worsen with fasudil treatment which may suggest that a low amount of basal ROCK activity may be necessary to maintain vascular homeostasis. In a large prospective study of 751 patients, elevated ROCK activity predicted cardiovascular events such as stroke, coronary revascularization, or death from any cardiovascular cause, but interestingly was not associated with an increased risk of MI (Kajikawa et al. 2014). Finally, in patients with concomitant CAD/PAD, leukocyte ROCK activity is elevated beyond the level found in patients with CAD alone, suggesting that ROCK may be a potential biomarker of atherosclerotic burden in patients with polyvascular disease (Dong et al. 2013a).

Interestingly, ROCK may also play a role in the development of vasospastic angina. In one study, leukocyte ROCK activity correlated directly with severity of coronary vasospastic angina in human patients (Hung et al. 2012). In a small study of patients with acetylcholine-induced vasospastic angina, fasudil significantly reduced coronary artery spasm and resultant myocardial ischemia (Masumoto et al. 2002). This evidence is consistent with a role for ROCK in multiple forms of coronary disease in humans.

Stroke

Ischemic stroke is a leading cause of death worldwide. While several agents have shown efficacy in reducing infarct size in animal experiments, translation of these effects to human patients has proven elusive. The neuronal injury and tissue loss associated with stroke are largely due to mechanisms of secondary injury such as inflammation and endothelial dysfunction which worsen the neurologic outcome in patients with ischemic stroke (Shibuya et al. 2005). Therefore, the reduction of such secondary injury mechanisms could improve clinical outcomes.

Several studies have confirmed a role of ROCK in platelet activation both by regulating phosphatidylinositol 4-phosphate 5-kinase (PIP5K) (Yang et al. 2004), a key regulator of actin dynamics, and by increasing MLC phosphorylation through inhibition of MLC phosphatase (MLCP) which leads to increased actin/myosin interaction and platelet contraction (Ono et al. 2008). Further, ROCK-dependent

phosphorylation of MLC increases adhesion of platelets to fibrinogen, promoting thrombus propagation (Leng et al. 1998). Through positive feedback, activated platelets release LPA (Shimada and Rajagopalan 2010), sphingosine-1-phosphate (S1P) (Hemmings et al. 2006), and platelet-derived growth factor (PDGF) (Akiyama et al. 2008) which in turn increase expression of ROCK.

In addition to its role in platelet activation, ROCK activity contributes to endothelial dysfunction and vascular inflammation in the pathogenesis of stroke. In cultured cells, ROCK regulates thrombin-induced endothelial permeability in concert with VEGF (Sun et al. 2006; Gavard and Gutkind 2008) and increases infiltration of the subendothelium by inflammatory cells, an action inhibited by lovastatin (Greenwood et al. 2003). In rodent models, pharmacologic ROCK inhibition reduces atherogenesis (Mallat et al. 2003), and knockdown of ROCK1 (but not ROCK2) results in reduced neointimal formation, pro-inflammatory adhesion molecule expression, and leukocyte infiltration following vascular injury from carotid artery ligation (Noma et al. 2008).

Additionally, ROCK mediates the inflammation that follows acute ischemic injury to the brain. Through its negative effect on eNOS expression (by decreasing eNOS mRNA expression and stability (Laufs and Liao 1998)), ROCK decreases endothelial NO bioavailability which results in increased expression of adhesion molecules critical for neutrophil adhesion to the vessel wall such as P-selectin and ICAM (Wang and Liao 2012). Further, ROCK stimulates ROS production through NADPH oxidase activation. In cultured neural tissue, inhibition of ROCK2 with fasudil reduces hypoxia/reoxygenation injury; decreases release of the inflammatory factors IL-1β, IL-6, and TNF; and increases the anti-inflammatory factor IL-10 (Ding et al. 2010). Similarly, treatment with fasudil reduces endothelial neutrophil recruitment and adhesion after ischemic/reperfusion injury in a rodent model and decreases infarct size (Satoh et al. 1999, 2001, 2008).

As the tissue loss from ischemic stroke occurs in large part due to post-infarct inflammation (Magnus et al. 2012), understanding the molecular mechanisms leading to inflammation in vascular and neural tissue is critical. While it is believed that ROCK has a direct regulatory effect on T-cell function, this action is incompletely understood. A growing body of evidence suggests that ROCKs regulate T-cell activation through effects on the actin cytoskeleton (Wang and Liao 2012) and through modulation of pro-inflammatory transcription factors such as JNK (Teramoto et al. 1997) and NF-κB (Perona et al. 1997) which both regulate T-cell function and activation. RhoA has been shown to control T-cell differentiation and survival as a downstream regulator of the TCR (Corre et al. 2001) and to upregulate production of IL-4, IL-10, and IFN-γ (Koprak et al. 1999; Schafer et al. 1999). Consistent with these findings, ROCK inhibition with Y-27523 inhibits actin-myosin interaction, decreases expression of inflammatory cytokines, and prevents TCR/CD3 complex aggregation in cultured T cells (Tharaux et al. 2003). In humans with ischemic stroke, leukocyte ROCK activity measured at 24 and 48 h after hospital admission is elevated compared with risk-matched control subjects (Feske et al. 2009) and independently predicts recurrent stroke (Cheng et al. 2014).

Modulation of the inflammatory response to stroke through inhibition of ROCK may be beneficial. ROCK inhibition by pretreatment with fasudil or Y-27632 significantly reduced infarct size in a rodent model of ischemic stroke in an eNOS-dependent manner (Shin et al. 2007). Further, pretreatment with fasudil increased cerebral blood flow, decreased infarct size, and improved neurologic deficit scores to an extent that correlated directly with the degree of ROCK inhibition and degree of eNOS expression and activity in brain and vascular cells (Rikitake et al. 2005a).

Studies in humans are limited; however, one promising multicenter, double-blind, placebo-controlled, phase III clinical trial did demonstrate that treatment with fasudil within 48 h of ischemic stroke led to improvements in neurologic function and clinical outcome (Shibuya et al. 2005). At the time of this publication, additional trials remain ongoing.

Genetic Variation in ROCK and Risk of Ischemic Stroke

In the past decade, twin (Brass et al. 1992; Bak et al. 2002) and familial studies (Jerrard-Dunne et al. 2003; Flossmann et al. 2004) have suggested a strong genetic component for the risk of ischemic stroke. In 1975, a twin study investigating the cause of death did not show a difference in concordance for fatal stroke between monozygotic and dizygotic twins (de Faire et al. 1975). However, a later study using mailed questionnaires showed a fivefold increase in the risk of stroke among monozygotic twins compared with dizygotic twins (Brass et al. 1992). A more recent study showed increased concordance for stroke, death, or hospitalization for stroke in monozygotic twins (Bak et al. 2002). Furthermore, a meta-analysis confirmed that monozygotic twins had higher concordance for stroke than dizygotic twins (odds ratio, 1.65; 95 % confidence interval, 1.2–2.3) (Flossmann et al. 2004). A number of familial studies as well as a meta-analysis showed that a positive family history was a risk factor for stroke (Flossmann et al. 2004). Many studies using either a candidate gene approach or genome-wide association studies (GWAS) have been conducted to identify susceptible genes. However, a lot of findings are inconsistent (Bevan et al. 2012; Sharma et al. 2013). A large number of gene variants identified failed to be replicated in GWAS (Bevan et al. 2012). Furthermore, several gene variants identified from GWAS were not confirmed in different studies. For example, a GWAS study showed that two single-nucleotide polymorphisms (SNPs) near the *NINJ2* (Ikram et al. 2009) and *WNK1* genes were associated with the risk of ischemic stroke. However, the Wellcome Trust Case Control Consortium failed to replicate the results (International Stroke Genetics and Wellcome Trust Case-Control 2010). Many factors may have contributed to the inconsistence, including quality control, errors in genotyping, and variable coverage of human genome with GWAS (Sharma et al. 2013). Not surprisingly, the genetic basis for ischemic stroke remains largely unknown.

In a recent Women's Genome Health Study (WGHS), our group identified 7 out of 8 searched tagging SNP (*t* SNPs) in *ROCK1* that are significantly associated with risk of ischemic stroke (Zee et al. 2014). Some of the *t* SNPs are located in the introns near the ATP-dependent kinase domain while others are located in the 5′ gene regulatory regions. In contrast, none of the *t* SNPs in *ROCK2* was found to have a significant association with the risk of stroke. Furthermore, this study revealed that three *t* SNPs (rs7007884, rs17683288, rs4876268) in *ARHGEF10* were associated with stroke susceptibility. *ARHGEF10* encodes a guanine nucleotide exchange factor 10 that was found to activate RhoA. In a Japanese study, a different SNP (rs2280887) in *ARHGEF10* was found to be associated with risk of ischemic stroke (Matsushita et al. 2010). This discrepancy may represent different susceptibility to *ARHGEN10* gene variation among different ethnic populations. Taken together, the findings suggest a potential genetic contribution along the RhoA-ROCK signaling pathway in the pathogenesis of ischemic stroke; however, confirmation of these findings in future prospective studies may open up new avenues for stroke prevention.

Diabetes Mellitus and Its Complications

Diabetes mellitus and the metabolic syndrome are associated with impairment of insulin signaling as well as numerous cardiovascular complications ranging from atherosclerosis to erectile dysfunction (Grundy et al. 2002). A growing body of evidence has implicated RhoA and ROCK in the development of these complications through various pathways. However, seemingly conflicting evidence currently confounds our understanding of the true role of ROCK in the development of cardiovascular complications in diabetes.

ROCK is a key regulator in insulin signaling and is known to phosphorylate insulin receptor substrate-1 (IRS-1). However, the net effect of ROCK activity on insulin signaling is controversial as in vitro and in vivo studies have yielded conflicting results (Sawada and Liao 2014). Most studies favor a detrimental role of ROCK activity on insulin signaling. Studies in multiple cell types including H9c2 rat cardiac myoblasts (Lim et al. 2007), C2C12 mouse myoblasts (Lim et al. 2007), and rat VSMCs (Begum et al. 2002) demonstrate that ROCK phosphorylates IRS-1 at Ser307 which impairs activation of PI-3K. However, in 3T3-L1 adipocytes and L6 myotubes, ROCK-mediated phosphorylation of IRS-1 at Ser632/635 enhances insulin signaling by facilitating tyrosine phosphorylation of IRS-1 (Furukawa et al. 2005). Additionally, ROCK1 knockout in both of these cell types impairs glucose transport, glucose transporter 4 translocation, and insulin-induced IRS-1 phosphorylation while overexpression of ROCK1 induces insulin hypersensitivity (Chun et al. 2012). Finally, ROCK inhibition with both fasudil and Y-27632 increases insulin mRNA expression in beta cell-derived HIT-T15 cells (Nakamura et al. 2006). Therefore, the sum of in vitro evidence seems to suggest that the effect

of IRS-1 phosphorylation by ROCK is at the very least context dependent and may vary by cell type or physiologic situation.

Evidence from in vivo studies is equally complex. While ROCK inhibition with fasudil improves insulin signaling and glucose tolerance in obese Zucker rats (Kanda et al. 2006), inhibition of ROCK with Y-27632 promotes insulin resistance by inhibiting insulin-mediated glucose uptake in skeletal muscle (Furukawa et al. 2005). Consistent with these findings, global ROCK1 deficiency causes insulin resistance in a mouse model (Lee et al. 2009) suggesting one possible explanation for the recent finding that statin therapy is associated with a small increased risk for the development of diabetes.

ROCK activity has also been implicated in the development of the metabolic syndrome characterized by central adiposity, dyslipidemia, hypertension, and glucose intolerance. In a mouse model of high-fat diet-induced metabolic syndrome, ROCK activity in adipose tissue was elevated compared with mice fed normal chow, and ROCK inhibition with fasudil reduced weight gain in HFD-fed mice (Hara et al. 2011). Similarly, constitutive activation of RhoA in 3T3-L1 adipocytes increases expression of the inflammatory cytokines PAI-1 and MCP-1, an effect reversed by Y-27632, suggesting that the Rho/ROCK pathway positively regulates expression of inflammatory cytokines in adipocytes (Nakayama et al. 2009). This evidence supports the notion that the Rho/ROCK pathway induces adipogenesis and inflammation and may be beneficial as a therapeutic target.

However, the Rho/ROCK pathway may also be required for normal growth and development. Constitutive Rho/ROCK activation in mice due to p190-B Rho-GTPase-activating protein deficiency impairs adipogenesis and favors myogenesis, an effect reversed by inhibition of ROCK with Y-27632 (Sordella et al. 2003). Additionally, ROCK activation enhances recovery after skeletal muscle injury, induces myogenesis in cultured C2C12 cells, and inhibits insulin-induced adipogenesis in 3T3-L1 preadipocytes (Bryan et al. 2005). Interestingly, deletion of ROCK2, but not ROCK1, enhances adipogenic differentiation in mouse embryonic fibroblasts, implicating the effect of ROCK2 as an important inhibitor of adipogenesis (Noguchi et al. 2007). Taken together, these findings highlight the context- and tissue-dependent role of ROCK1 and ROCK2 in the development of the metabolic syndrome and glucose intolerance.

ROCK activity has also been implicated in the development of endothelial dysfunction, a precursor to cardiovascular complications associated with diabetes in both animal models (Okon et al. 2003) and in humans (Tabit et al. 2010). A growing body of literature suggests that Rho/ROCK signaling is an important contributor to the pathogenesis of endothelial injury in diabetes. Specifically, advanced glycation end products (AGEs) activate the receptor RAGE which can complex with RhoA to induce ROCK activation. This interaction causes cytoskeletal rearrangement by actin filaments and leads to the endothelial hyperpermeability phenotype of diabetes (Hirose et al. 2010). In cell culture models, ROCK activity has been shown to be responsible for hyperglycemia-induced plasminogen activator inhibitor-1 (PAI-1) overexpression through an NF-κB-dependent mechanism (Iwasaki et al. 2008). In separate studies, hyperglycemia has been shown to increase

ROCK activity through a mechanism involving PKC which leads to increased oxidative stress, an effect abolished by ROCK inhibition with fasudil or Y-27632 (Rikitake and Liao 2005). Finally, in a mouse model of streptozocin-induced diabetes, ROCK2 knockout is sufficient to prevent diabetes-associated impairment of corpus cavernosal vascular relaxation (Toque et al. 2013). These findings suggest a critical link between ROCK activity, endothelial inflammation, and the numerous complications associated with diabetes.

While data from humans regarding the role of ROCK in the development of diabetes-induced endothelial dysfunction is limited, PKC has been shown to be overexpressed in endothelium from diabetic humans and is associated with impaired insulin signaling, activation of NF-κB, elevation of ROS, and worsening endothelial function (Tabit et al. 2013). In this study, inhibition of PKC improved endothelial insulin signaling suggesting that inhibition of ROCK activity through PKC could be a potential therapeutic target for diabetic vascular disease. In a study of Taiwanese patients with metabolic syndrome, ROCK activity was increased in the cohort with metabolic syndrome versus healthy controls and was associated with an increase in BMI, waist circumference, fasting serum glucose, hsCRP, and triglyceride levels and correlated with the severity of metabolic syndrome (Liu et al. 2007). Taken together, these data suggest that ROCK is an important player in the development of metabolic syndrome.

Myocardial Hypertrophy, Fibrosis, and Heart Failure

Myocardial hypertrophy and fibrosis are hallmarks of several pathophysiological mechanisms. Myocardial hypertrophy can occur in a pressure-dependent manner in disease states such as hypertensive heart disease and valve stenosis or in a pressure-independent manner due to hypertrophic cardiomyopathy or amyloidosis. Myocardial fibrosis typically follows ischemic injury to the heart or chronic inflammation such as myocarditis, pericarditis, or sarcoidosis. While the origins of each of these patterns of myocardial injury differ, both frequently lead to progressively worsening heart failure. Recent evidence implicates ROCK activity in the development of both hypertrophy and fibrosis in patients with heart failure.

In animal models, ROCK inhibition with fasudil prevents negative myocardial remodeling in salt-sensitive hypertensive rats (Takeshima et al. 2012) and similarly prevents negative remodeling and fibrosis in mice treated with transverse aorta constriction (Li et al. 2012). Similarly, fasudil administration attenuates angiotensin II-induced myocardial hypertrophy (Higashi et al. 2003) and reduces Adriamycin-induced myocardial fibrosis in rat models (Wang et al. 2011). In humans, activity and expression of ROCK1 and ROCK2 were elevated in patients admitted to the hospital with heart failure versus disease controls and normal subjects, and an elevation of ROCK activity added to NT-pro-BNP was an independent predictor of mortality (Dong et al. 2012). Interestingly, while ROCK1 deletion does not prevent the development of cardiac hypertrophy (Rikitake et al. 2005b), it does reduce the development of systolic dysfunction (Shi et al. 2008), fibrosis (Rikitake

et al. 2005b), and cardiomyocyte apoptosis (Chang et al. 2006), suggesting a complex balance between the adaptive and pathologic roles of ROCK in the myocardium. Further investigation is needed to better describe this complex role of ROCK in myocardial hypertrophy, fibrosis, and heart failure.

Hypertension

Increased ROCK activity has been demonstrated in animal models of hypertension (Moriki et al. 2004) as well as hypertensive human patients (Masumoto et al. 2001). RhoA/ROCK activity appears to correlate with activation of the renin/angiotensin/aldosterone system (Guilluy et al. 2010), a pathway known to contribute to the pathophysiology of hypertension in humans. However, the exact role of ROCK in the regulation of blood pressure is unclear as animal studies have yielded conflicting results. One study in ROCK1 haploinsufficient mice demonstrated similar blood pressures as WT littermates (Rikitake et al. 2005b), while a more recent study showed reduced baseline blood pressure and attenuated diabetes-induced endothelial dysfunction in ROCK1 knockout mice (Yao et al. 2013). While pharmacologic ROCK inhibition reduces vascular smooth muscle contractility (Chan et al. 2009; Yao et al. 2013) and improves endothelial function (Tsounapi et al. 2012), studies showing that ROCK inhibition lowers blood pressure are currently lacking.

Pulmonary Arterial Hypertension

Pulmonary arterial hypertension (PAH) is a debilitating progressive disease which carries significant morbidity and mortality. Despite recent advances in the treatment of PAH with agents such as prostacyclin analogues, endothelin receptor antagonists, and phosphodiesterase-5 inhibitors, therapy is often ineffective, and prognosis remains poor. There is emerging evidence that ROCK is involved in the pathogenesis of PAH (Barman et al. 2009), and therefore the action of the ROCK inhibitor fasudil on the pulmonary vasculature is of great clinical interest. ROCK activity in circulating neutrophils and ROCK expression in lung tissue are elevated in human patients with PAH and ROCK activity correlates with severity and duration of PAH (Do e et al. 2009). Further studies have demonstrated a link between the 5-HT transporter and ROCK activity in humans with PAH as well as a mouse model. RhoA/ROCK activity was significantly higher in lungs, platelets, and pulmonary artery smooth muscle cells from patients with PAH and was accompanied by a strong increase in the binding of 5-HT to RhoA. Treatment with fluoxetine (a 5-HT inhibitor) prevented RhoA/ROCK activation and decreased proliferation of SMCs from patients with PAH. Finally, both fasudil and fluoxetine limit progression of PAH in a mouse model (Guilluy et al. 2009).

While studies investigating the clinical utility of fasudil in PAH are very limited, several small cohorts have demonstrated mild reductions in pulmonary artery pressure in human patients with PAH after fasudil administration either through

intravenous (Fukumoto et al. 2005; Ishikura et al. 2006) or inhaled (Fujita et al. 2010) routes of administration. However, there are currently no large clinical trials available, and data regarding mortality impact has not been published.

ROCK Inhibitors in the Treatment of Cardiovascular Disease in Humans

As discussed above, pharmacologic inhibition of ROCK with fasudil or the more novel compound Y-27632 is sufficient to abrogate many of the downstream effects of ROCK activity. Clinically, fasudil has been shown to prevent cerebral vasospasm after subarachnoid hemorrhage in humans (Suzuki et al. 2008), and both fasudil and Y-27632 inhibit atherogenesis and arterial remodeling after vascular injury (Zhou and Liao 2009). While large clinical trials are lacking, fasudil has shown promise in small human studies in the treatment of systemic hypertension, pulmonary hypertension, vasospastic angina, stable effort angina, stroke, and chronic heart failure (Sawada and Liao 2014). Similarly, fasudil has been shown to prevent myocardial hypertrophy and fibrosis in a rodent model (Ho et al. 2012) although human studies are lacking. Additionally, a novel ROCK inhibitor SAR407899 has been recently developed with reported superior efficacy in ROCK inhibition though available data is limited (Lohn et al. 2009). However, all current ROCK inhibitors nonspecifically inhibit both ROCK isoforms. As we have discussed above, ROCK1 and ROCK2 have vastly different roles in physiology and pathophysiology. Therefore, currently the pharmacologic efficacy of ROCK inhibitors is limited by the inability to target one isoform over the other.

Recently, a new ROCK inhibitor SLx-2119 has been characterized which is 100-fold more selective for ROCK2 than ROCK1. In a single study of MCA stroke in a mouse model, SLx-2119 significantly increased cortical blood flow after MCA occlusion without the drop in systemic blood pressure typical of nonselective ROCK inhibitors (Lee et al. 2014). However, much further study is necessary to determine the efficacy and safety of this compound in humans and its role in treating cardiovascular disease.

HMG-CoA reductase inhibitors, commonly known as statins, are a family of molecules that inhibit the rate limiting step of cholesterol biosynthesis, the conversion of HMG-CoA to L-mevalonic acid (Istvan and Deisenhofer 2001). This action reduces cholesterol synthesis in the liver and upregulates hepatic LDL receptor expression, thereby enhancing clearance of LDL cholesterol from the blood. Additionally, statins inhibit the production of downstream isoprenoid intermediates such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) (Goldstein and Brown 1990) which serve as crucial membrane attachment sites for small GTPase proteins such as Rho, Rac, Rap, and Ras (Van Aelst and D'Souza-Schorey 1997). Statins thereby inhibit membrane targeting and activation of Rho which prevents the subsequent activation of ROCK (Fig. 3). Through this mechanism of ROCK inhibition, statins have been postulated to play a key role in multiple beneficial cardiovascular processes independent of their lipid-lowering effects

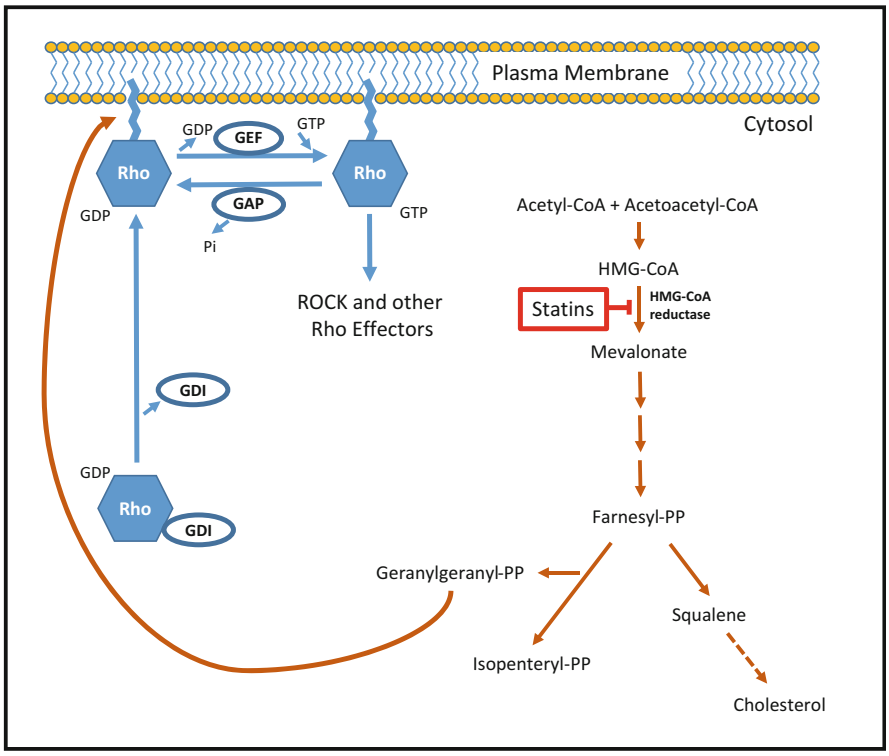

Fig. 3 Regulation of Rho small GTP-binding proteins by the cholesterol biosynthesis pathway and isoprenoids. Rho family proteins require GGPP for their anchoring to the cell membrane where GTP binding activates the small GTPase. Statins inhibit HMG-CoA reductase leading to a decrease in GGPP biosynthesis and a decrease in isoprenylation of Rho. *GAP* GTPase-activating protein, *GDI* GDP dissociation inhibitor, *GEF* guanine nucleotide exchange factor, *GGPP* geranylgeranyl pyrophosphate, *HMG-CoA* 3-hydroxy-methylglutaryl-coenzyme A, *ROCK* Rho-associated coiled-coil kinase

(Zhou and Liao 2010). Known as "pleiotropic effects," these beneficial actions of statins may be due in large part to their inhibition of Rho isoprenylation and subsequent inhibition of ROCK.

Potential Applications to Prognosis, Other Diseases, or Conditions

Elevated ROCK activity is a predictor of poor outcomes in several forms of cardiovascular disease. ROCK activity is known to correlate with Framingham risk score and multiple risk factors for cardiovascular disease including body mass index, systolic blood pressure, LDL cholesterol, and impairment in endothelial function as measured by flow-mediated dilatation (Soga et al. 2011). In a large

study of Japanese patients, elevated ROCK activity was an independent predictor of negative cardiovascular outcomes such as stroke and also predicted the need for coronary revascularization through bypass surgery or PCI (Kajikawa et al. 2014). In this study, high ROCK activity also predicted the incidence of first major cardiovascular events and was associated with a significantly higher risk of death from cardiovascular disease at 5 years. While several small studies have produced similar findings, this represents the largest population-based study of the prognostic effect of ROCK activity on cardiovascular outcomes.

In addition to its role in the pathogenesis in multiple forms of cardiovascular disease, ROCK has been extensively studied in other disease types such as cancer and pulmonary fibrosis, and emerging evidence may support a role for ROCK in neurocognitive disorders such as Alzheimer's disease. In cultured malignant cell lines (Rosel et al. 2008) and in tumor samples from human patients with various cancers (Kamai et al. 2002, 2003), ROCK activity and expression is elevated. Further, numerous known downstream targets of ROCK have been shown to control oncogenic transformation and cell growth to varying degrees. For example, c-Jun N-terminal kinase (JNK)-interacting protein 3 which inhibits the action of JNK is phosphorylated and inactivated by ROCK (Schofield and Bernard 2013). Phosphatase and tensin homologue (PTEN), another inhibitor of JNK signaling, is also phosphorylated and inactivated by ROCK. PTEN inactivation is associated with the development of melanoma. Therefore, this evidence suggests that ROCK may play a role in the repression of key cell cycle regulators and may be involved in oncogenesis.

Other studies have implicated ROCK in the development of pulmonary fibrosis after lung injury. ROCK2 is activated in various pulmonary cell types from animal models of lung fibrosis (Shimizu et al. 2014). Additionally, ROCK inhibition with fasudil reduced bleomycin-induced pulmonary fibrosis and pulmonary hypertension in mice (Bei et al. 2013), and similar treatment induced myofibroblast apoptosis and inhibited fibroblast to myofibroblast transition in animal models and cultured cells treated with TGF-β (Zhou et al. 2013). Taken together, these preliminary studies suggest a role for ROCK in the development of pulmonary fibrosis.

Finally, emerging evidence may implicate ROCK in the development of neurocognitive disorders. In one interesting study, inhibition of the RhoA/ROCK pathway improved spatial learning and working memory in a rodent model (Huentelman et al. 2009), suggesting a possible role for ROCK inhibitors in the treatment of Alzheimer's disease. However, much further study is needed to determine the precise role of ROCK in the mechanism of learning and the potential for therapeutic benefit.

Conclusion

Increasing evidence from clinical studies and animal models highlight the importance of ROCK in the pathogenesis of multiple forms of cardiovascular disease and other pathologic states. Therefore, Rho/ROCK presents a promising target for

pharmacologic inhibition in the treatment of cardiovascular disease. Indeed, many of the cholesterol-independent pleiotropic effects of statins may in fact be due to their inhibition of isoprenoid synthesis and subsequent inhibition of the Rho/ROCK pathway. However, despite a wealth of data in animal models of disease, little evidence is available from human trials. Further, little is known regarding the downstream targets of ROCK in the context of inflammatory and metabolic disorders including atherosclerosis and diabetes. Also, an increased knowledge of the isoform-specific action of ROCK1 and ROCK2 as well as isoform-specific inhibitors would be clinically useful in treating patients with cardiovascular disease.

Summary Points

- Rho-associated coiled-coil kinase (ROCK) is a downstream effector of Rho and regulates the actin cytoskeleton.
- ROCK phosphorylates and inhibits myosin light chain (MLC) phosphatase which increases phosphorylation of MLC.
- ROCK regulates numerous cellular functions such as division, death, and movement.
- The ROCK molecule assumes a closed (inactive) conformation unless bound to Rho-GTP. When Rho-GTP binds to the ROCK Rho-binding domain, ROCK assumes an open (active) conformation.
- Statins prevent production of geranylgeranyl pyrophosphate which links Rho to the plasma membrane. By preventing membrane linking, statins inhibit the action of Rho and the activation of ROCK, thus exhibiting "pleiotropic" effects.
- ROCK activity has been implicated in the development of numerous forms of cardiovascular disease including stroke, atherosclerosis, hypertension, pulmonary arterial hypertension, and myocardial fibrosis.
- ROCK may contribute to the development of diabetes mellitus and its complications.
- Fasudil is the best-studied pharmacologic inhibitor of ROCK. However, fasudil nonselectively inhibits both ROCK isoforms, limiting its clinical utility. Newer agents with more isoform selectivity may prove more clinically useful.

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Polymorphisms in the Vitamin D Pathway in Relation to 25-Hydroxyvitamin D Status and Cardiovascular Disease Incidence: Application to Biomarkers

33

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Contents

Abstract

Vitamin D deficiency has become a globally acknowledged problem whose impact on societies has proven to surpass all medical expectations. Vitamin D is no longer peerlessly associated with bone diseases. In fact, collaborations of clinicians and researchers have yielded the undeniable truth, that is, the affiliation of this unconventional vitamin with diseases that are currently grasping the media's attention like autoimmune diseases and cancers. Having established the importance of this phenomenon, assuming complete understanding of the association of vitamin D with one of the leading causes of death in the world,

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_23

cardiovascular disease, is only mildly precise. Observational studies tend to highlight the association of low vitamin D levels with various forms of cardiovascular disease as well as with the risk factors associated, whereas interventional studies have been conflicting. Nonetheless, in vitro studies have identified the presence of nuclear vitamin D receptors in the cardiovascular system in cells such as cardiomyocytes and endothelial cells, thereby warranting cardiovascular actions. Moreover, recent studies have demonstrated the ability of vitamin D to beneficially modulate effectors of the cardiovascular system such as the renin-angiotensin-aldosterone system and the nitric oxide system. While there appears to be abundance in the number of publications on the epidemiological and mechanistic association of the vitamin with the disease, studies aiming to investigate the genetic component of the relationship are sparse. Recent genome-wide association studies have identified single nucleotide polymorphisms (SNPs) in genes encoding proteins involved in the vitamin D pathway, whether synthesis, metabolism, or elimination, that are associated with circulating levels of 25-hydroxyvitamin D [25(OH)D], the biomarker of vitamin D status, and thus it is conceptualized that such SNPs may act as novel genetic markers for cardiovascular disease since the disease has been associated with low levels of 25(OH)D. Several studies have investigated this hypothesis, yielding both positive and negative associations, highlighting the need for further investigations into the proposed triangular relationships between the SNPs, 25(OH)D levels, and the disease, which would spawn sound evidence prompting or discouraging professionals to extrapolate the findings to clinical genetic testing.

Keywords
Vitamin D • Cardiovascular disease • SNP • Biomarkers • Vitamin D genetics

Abbreviations

$1,25(OH)_2D$	1,25-Dihydroxyvitamin D/calcitriol
25(OH)D	25-Hydroxyvitamin D
7-DHC	7-Dehydrocholesterol
ADMA	Asymmetric dimethylarginine
CVD	Cardiovascular disease
CYP	Cytochrome P450
eNOS	Endothelial nitric oxide synthase
GWAS	Genome-wide association study
MI	Myocardial infarction
NO	Nitric oxide
RAAS	Renin-angiotensin-aldosterone system
RCT	Randomized controlled trial
ROS	Reactive oxygen species
SNP	Single nucleotide polymorphism
UV-B	Ultraviolet-B
VDR	Vitamin D receptor
VDRG	Vitamin D receptor gene

Key Facts of Vitamin D

- Vitamin D is a fat-soluble vitamin that exists in two forms, vitamins D2 (ergocalciferol) and D3 (cholecalciferol).
- Vitamin D exerts its classical skeletal effects via increasing calcium absorption from the intestine, increasing mobilization from bones, and increasing renal tubular reabsorption.
- The majority of the daily vitamin D requirement comes from photosynthesis in the skin, with exogenous contributions through certain foods and supplementation.
- 25(OH)D is the clinical biomarker of vitamin D status, whereas $1,25(OH)_2D$ is the active form of the vitamin.
- Main sites of metabolism are the liver and kidneys.
- Dietary reference intake of vitamin D varies between countries and across different age groups within a population.

Definitions

Cardiovascular disease A class of diseases involving the heart and blood vessels.

Cytochrome P450 A class of monooxygenases that is utilized by the human body for the synthesis and metabolism of endogenous substances, such as cholesterol and vitamin D, respectively, detoxification of foreign substances, and drug metabolism.

Genome-wide association study A case-control study that examines the association of many common SNPs with various traits/diseases. Such studies are conducted on a "genome-wide" scale, hence the naming, thereby encompassing possibly millions of SNPs in a single study.

Genotyping The process by which the exact DNA sequence is determined.

Randomized controlled trial A clinical study that assesses the effect of a given medication on a cohort. It provides the strongest clinical evidence since randomization of subjects into intervention and placebo groups minimizes selection and allocation bias.

Response element Short DNA sequences in promoters of genes to which transcription factors bind and regulate the process of transcription.

Restriction enzymes Restriction endonucleases that were originally discovered in the 1970s in prokaryotes that endogenously use them as protection from foreign DNA. Several uses have been characterized for this class of enzymes in biological research, among which is their use as an efficient genotyping technique.

Single nucleotide polymorphism (SNP) A variation between individuals of a specific population at a certain nucleotide in their DNA. SNPs may have an effect on phenotypes, response to treatment, be susceptible to disease, or have no effect at all.

Transcription factor Protein that controls the transcription of certain genes by binding to defined DNA sequences and, with the help of other proteins, namely, co-regulators, promotes or blocks the recruiting of RNA polymerase.

Vitamin A vital, organic substance that is required by organisms in limited amounts. Vitamins are generally not produced in sufficient quantities by the body and are therefore sought after exogenously.

Introduction

When Adolf Windaus discovered vitamin D early in the twentieth century and was subsequently awarded the Nobel Prize, medical professionals worldwide thought that a bulletproof cure for rickets had been discovered, which left them contented with this life-saving property the substance was proven to possess. However, looking back at those times from the twenty-first century, one realizes that associating this molecule solely with bone health is nothing short of a colossal, scientific understatement.

Using the search engine PubMed to access the MEDLINE database and "vitamin D" as a keyword, one finds a plethora of publications ranging from clinical trials and observational studies to in vitro experiments and animal studies. According to the same search, the number of publications on this topic seems to have surpassed doubling over the past decade, with 1,258 manuscripts published in 2000, and 2,717 by 2010 (Fig. 1), which begs to wonder the sudden appeal of this very simple molecule to scientists despite its initial discovery many years ago.

From an epidemiological standpoint, numerous observational reports have identified low circulating levels of vitamin D as an independent marker for a multitude of chronic diseases such as cancer and autoimmune and cardiovascular disease (CVD) (Holick 2007; Abu El Maaty and Gad 2013; Hossein-Nezhad and Holick 2013). However, in such cases, one is baffled by the possible "egg or chicken" scenario. In other words, is vitamin D deficiency a cause or a consequence of the diseases?

While longitudinal studies have backed up the possible causality by demonstrating the development of disease or worsening of symptoms with decreasing vitamin D levels (McAlindon et al. 1996; Yin et al. 2009), randomized controlled trials (RCTs), which provide the strongest lines of evidence, have been so far inconclusive and present some limitations, such as insufficient dosing and supplementation of subjects exhibiting normal serum vitamin D levels. Building on this premise, several large, well-designed RCTs or "mega-trials" are currently under way to determine the clinical efficacy of supplemental vitamin D on various outcomes, such as the ViDA

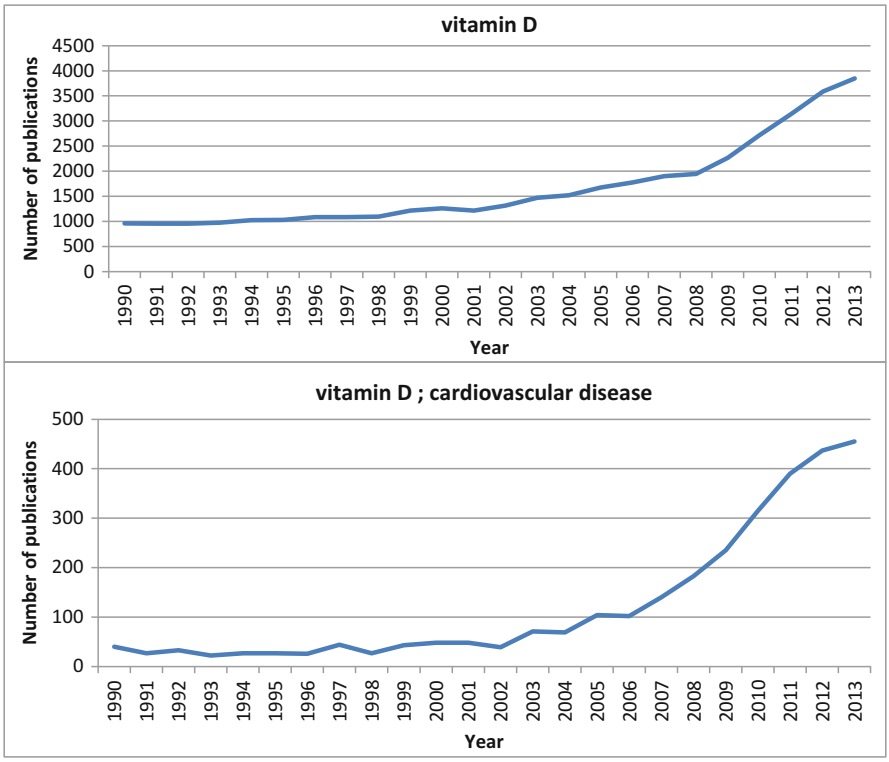

Fig. 1 Number of publications per year, from 1990 to 2013, according to PubMed, with "vitamin D" (*top*) or "vitamin D, cardiovascular disease" (*bottom*) as keywords. An exponential rise in the number of publications over time is apparent in both charts. Although propositions of a relationship between vitamin D and CVD or the cardiovascular system date back to the 1980s, research in this area remained stagnant for almost a decade, before picking up pace and providing substantial epidemiological and molecular evidence on the association

study that aims to provide answers for the vitamin D-CVD predicament by 2016 (Scragg 2011). Researchers anticipate a final clinical verdict on the apparent "panacea" in the coming years.

Vitamin D is a fat-soluble vitamin, with well-documented supra-skeletal abilities besides the classical calcium-regulating properties, such as regulation of cellular proliferation, immunomodulation, and cardioprotection (Holick 2007). Humans acquire their daily requirements of this vitamin mainly from sunlight, with further additions obtained from fortified foods as well as natural sources, such as fatty fish and mushrooms (Fig. 2).

Vitamin D exists in two forms, cholecalciferol (vitamin D3) andergocalciferol (vitamin D2), which undergo the same metabolizing steps and only differ in the sources, where the first is photosynthesized in the dermal and epidermal cells of the

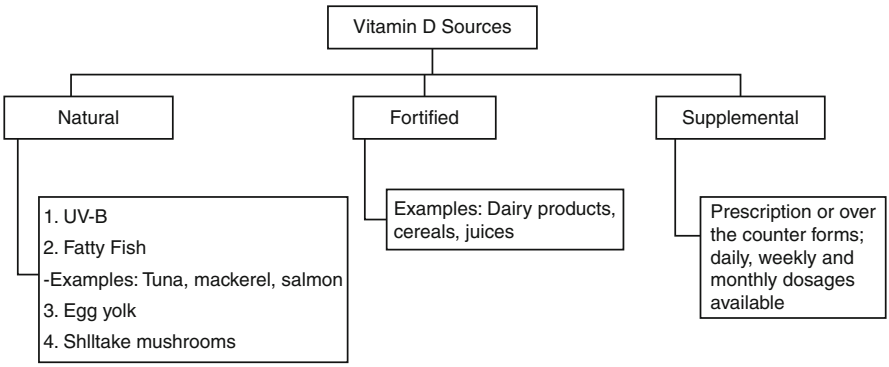

Fig. 2 Sources of vitamin D

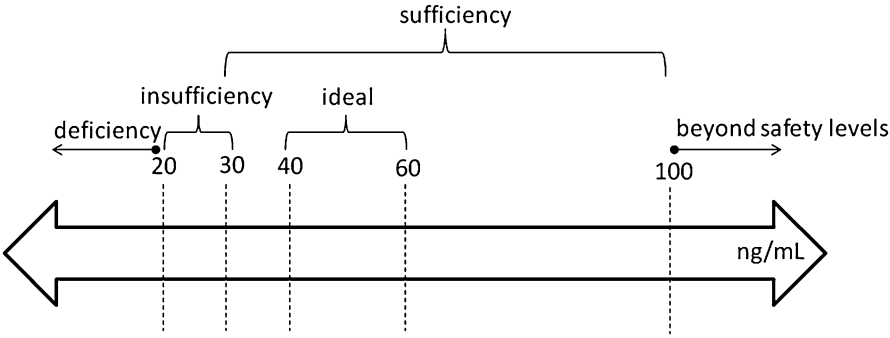

Fig. 3 Vitamin D status classification according to guidelines set by the US Endocrine Society. Values in ng/mL are converted to nmol/L by multiplying by 2.496. The ideal range is set to account for inter-assay variability, a substantial analytical issue observed in the clinical assessment of 25 (OH)D levels

skin upon exposure to the sun's ultraviolet-B (UV-B) radiation, whereas the other is obtained exclusively from the diet or via supplementation (Abu El Maaty and Gad 2013). The two forms have arguably the same potency in vivo and are equally prescribed to patients (Abu El Maaty and Gad 2013). The term vitamin D in this chapter refers to both forms.

According to the US Endocrine Society, subjects' 25-hydroxyvitamin D [25(OH)D] values, the clinical biomarker for vitamin D status, are classified as either deficient, insufficient, normal, or potentially toxic, as illustrated in Fig. 3 (Hossein-Nezhad and Holick 2013). Toxic levels of vitamin D are not reached with prolonged exposure to UV-B due to the natural homeostasis of cutaneous vitamin D biosynthesis, where vitamin D and previtamin D could be converted to inactive photoproducts thereby avoiding toxicity (Holick 2007).

The Vitamin D Biosynthetic Pathway: A Gateway to Vitamin D Genetics

Vitamin D biosynthesis in humans is initiated in the skin by the exposure of an intermediate of the cholesterol biosynthetic pathway, 7-dehydrocholesterol (7-DHC), to UV-B, yielding previtamin D3. Besides entering the vitamin D biosynthetic pathway, 7-DHC is converted to cholesterol by the corresponding reductase enzyme (DHCR7). Previtamin D3 undergoes thermal isomerization and is converted to the thermodynamically favorable vitamin D3, which then enters the circulation and is accompanied by vitamin D obtained from dietary sources (Abu El Maaty and Gad 2013).

Since vitamin D is a fat-soluble vitamin, it circulates the body bound to a binding protein, referred to as the vitamin D-binding protein that is coded for by the GC gene (Abu El Maaty and Gad 2013). The first metabolizing step this vitamin undergoes occurs in the liver, via actions of the enzyme vitamin D-25-hydroxylase, coded for not exclusively by the CYP2R1 gene, which yields the quantifiable form of the vitamin (Abu El Maaty and Gad 2013). The second metabolizing or "activating" step occurs to a large extent in the kidneys, via actions of the enzyme 25(OH)D-1-hydroxylase, coded for by the CYP27B1 gene, giving rise to the hormonally active form of the vitamin, 1,25-dihydroxyvitamin D [$1,25(OH)_2D$], also commonly referred to as calcitriol (Hossein-Nezhad and Holick 2013). Studies have illustrated the presence of mitochondrial CYP27B1 in a number of cell types, supporting the notion that vitamin D acts in both endocrine and autocrine manners. In other words, calcitriol could be produced in the kidneys and then travels to the intestine, for instance, to exert its actions, which is the classical route for most hormones; hence, endocrine or calcitriol is produced and exerts its actions in the target cell, hence autocrine (Abu El Maaty and Gad 2013).

Although rapid, non-genomic actions for vitamin D have been documented (Haussler et al. 2011), genomic effects of vitamin D seem to dominate this area of research, which are orchestrated by the nuclear vitamin D receptor (VDR), coded for by the VDR gene (VDRG). Upon binding of calcitriol, VDR heterodimerizes with the retinoid X receptor and translocates into the nucleus, where it binds to vitamin D response elements on promoters of target genes, influencing their transcription (Abu El Maaty and Gad 2013). In this case, the VDR acts as a transcription factor that appears to influence the expression of approximately several hundred genes, depending on the cell type, nonetheless impacting a substantial portion of the human genome (Ramagopalan et al. 2010; Hossein-nezhad et al. 2013).

Vitamin D metabolites, whether 25(OH)D or $1,25(OH)_2D$, are acted upon by a 24-hydroxylating enzyme, coded for by the CYP24A1 gene, yielding the water-soluble, excretable form calcitroic acid, which is eliminated in the kidneys (Holick 2007; Abu El Maaty and Gad 2013). A schematic overview of the pathway is depicted in Fig. 4.

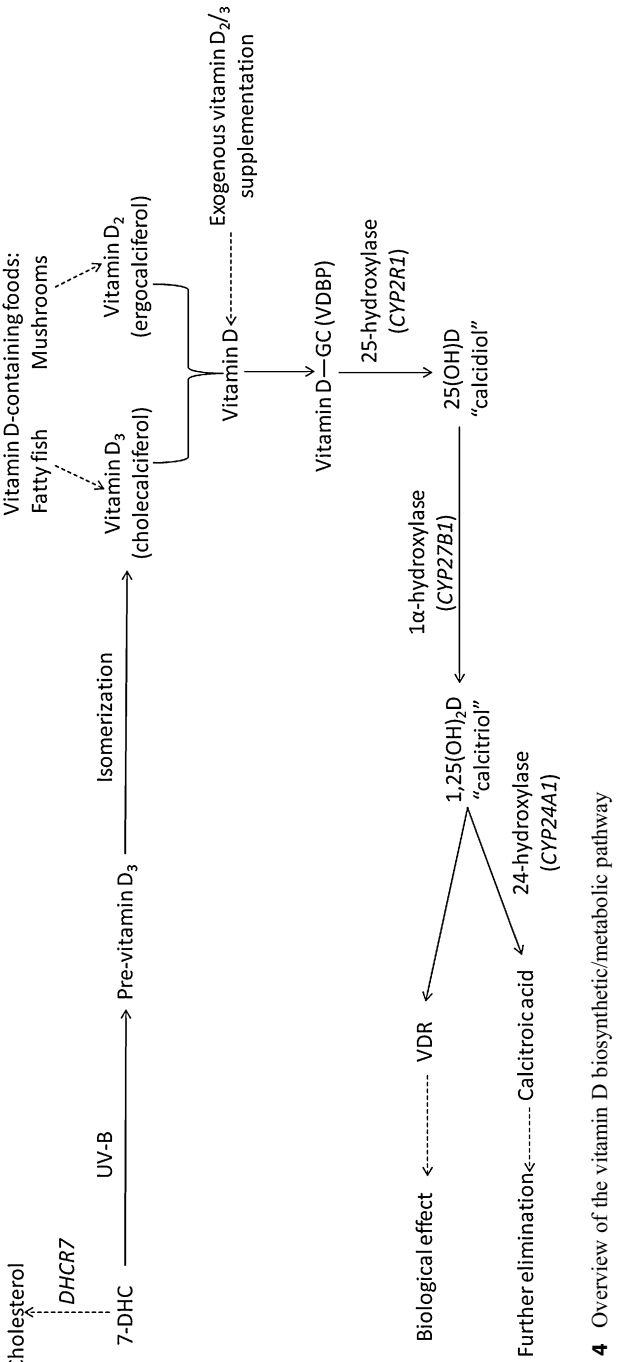

Fig. 4 Overview of the vitamin D biosynthetic/metabolic pathway

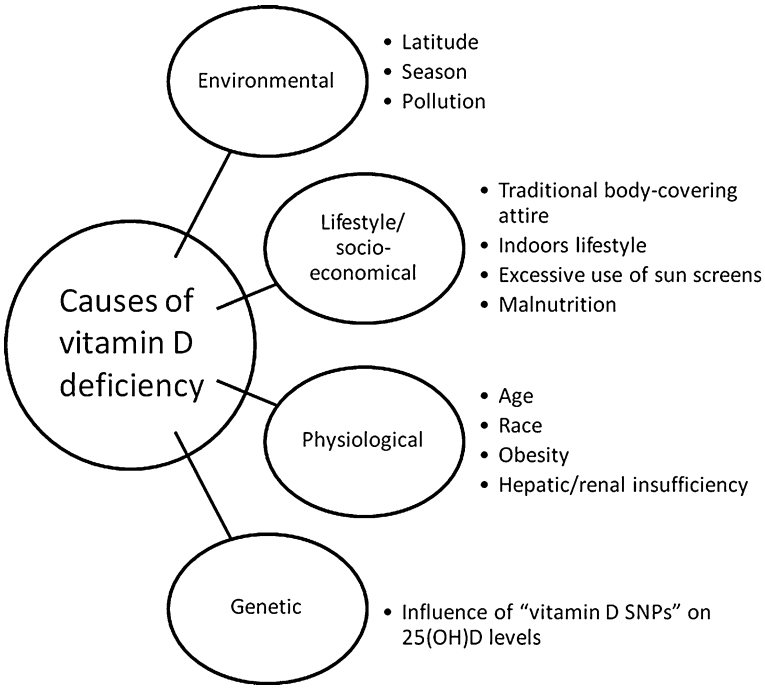

Fig. 5 Causes of vitamin D deficiency

Exploring the Genetic Determinants of Vitamin D Status

For many years, the factors governing circulating vitamin D levels were restricted to the geographical location, skin pigmentation, time of year, and several others described elsewhere (and summarized in Fig. 5) (Holick 2007); however, in 2010, results of two published genome-wide association studies (GWASs) shed light on a new factor, genetic polymorphism of genes encoding proteins in the vitamin D biosynthetic pathway (Ahn et al. 2010; Wang et al. 2010). Soon after that, results of those primary publications were replicated by different groups who conducted similar studies on various cohorts (Signorello et al. 2011; Zhang et al. 2012), which opened new avenues of research aimed to elucidate genetic predictors for vitamin D status, with the purpose of identifying groups at high risk for deficiency.

Furthermore, prospects of novel genetic biomarkers for disease were raised, based on possible triangular relationships between the identified vitamin D status-associated SNPs, 25(OH)D levels, and various disease phenotypes. Despite it being an intriguing, very ambitious notion, investigations of such relationships are not sufficient and have been predominantly conducted on subjects of European descent, such as the European Investigation into Cancer and Nutrition (EPIC) study (Kuhn et al. 2013), the Ludwigshafen Risk and Cardiovascular Health (LURIC) study (Trummer et al. 2013), and the Tromso study (Jorde et al. 2012). Table 1 highlights

Table 1 Genes in the vitamin D pathway, their genetic location, and SNPs in them implicated by previous studies as having an effect on 25(OH)D levels, CVD, or both

Gene	Region	SNPs of interest
DHCR7/NADSYN1	11q13.4	rs3829251
		rs1790349
		rs12785878
GC	4q13.3	rs2282679
		rs7041
CYP2R1	11p15.2	rs2060793
		rs10741657
CYP27B1	12q14.1	rs703842
		rs4646536
CYP24A1	20q13	rs6013897
VDRG	12q13.11	rs2228570
		rs1544410
		rs7975232
		rs731236

the candidate genes and SNPs of interest for further research based on results of conducted studies.

Additionally, in this regard, a recently published study elucidated possibly contrasting roles of vitamin D SNPs on 25(OH)D levels in subjects of distinct ethnicity, where the authors elucidated, among other results, that different SNPs were strongest associated with 25(OH)D in African and European Americans (AA and EA, respectively); however, both reported SNPs were mapped to the CYP2R1 gene (rs12794714 for AA and rs1993116 for EA) (Batai et al. 2014).

Although several attempts have been done to link vitamin D-related SNPs with CVD risk factors such as obesity (Vimaleswaran et al. 2013), hypertension (Wang et al. 2014), and type 2 diabetes (Ye et al. 2015), a clear connection with CVD hasn't been established, since studies aimed to elucidate such connection, i.e., with CVD incidence as the main outcome, are scarce. We herein discuss results of recently conducted studies in this area and highlight possible areas of future research.

Triangular Relationship Between the SNPs, 25(OH)D, and CVD

DHCR7/NADSYN1

The involvement of DHCR7 mutations with 25(OH)D levels has been overshadowed for many years by their classical involvement with the autosomal recessive disorder, Smith-Lemli-Opitz syndrome, abbreviated SLOS, which is also known as 7-dehydrocholesterol reductase deficiency.

As previously mentioned, the enzyme encoded by the DHCR7 gene catalyzes the final step of cholesterol biosynthesis, the conversion of 7-DHC to cholesterol. Additionally, this enzyme affects vitamin D3 photosynthesis in the skin, by controlling the amount of 7-DHC available to enter the vitamin D biosynthetic pathway. It is thus speculated that mutations in this gene may affect the function of the translated

protein, resulting in either higher or lower activity enzymes, which essentially determine vitamin D's share of 7-DHC (Abu El Maaty et al. 2014).

Recently reported findings on DHCR7 mutations have shown that this gene may have undergone positive selection to accommodate the early humans' migratory patterns away from the equator and toward the northern hemisphere, where the authors have identified several SNPs in the locus with significantly different allelic distributions between populations that are geographically distinct (Kuan et al. 2013). It was subsequently hypothesized that individuals located far from the equator may have harbored mutations in this gene that favor cutaneous vitamin D3 biosynthesis, possibly by coding a less active form of the enzyme, thereby reducing the flux of 7-DHC through the cholesterol biosynthetic pathway, and increasing the amounts of 7-DHC available for the vitamin D pathway. This hypothesis serves as a possible explanation for the compensating mechanism that was imposed through positive selection due to decreased amounts of sunlight available in northern regions.

Although several studies have replicated the findings of the ability of SNPs in the DHCR7 gene to predict 25(OH)D levels, reports on triangular associations including the third partner of the triad, CVD, are sparse. Our lab has recently conducted a pilot study investigating this hypothesized link; however, we found a lack of connection between the two investigated SNPs in the DHCR7/NADSYN1 locus (rs12785878 and rs1790349) and CAD, yet we also observed the ability of both SNPs to predict 25(OH)D concentrations (Abu El Maaty et al. 2014).

Several months prior to publication of our findings, results of three independent studies involving European subjects had shown a lack of association between the DHCR7 SNP rs12785878 and cardiovascular mortality and incident myocardial infarction (MI), yet the ability of the SNP to predict 25(OH)D levels is observed (Jorde et al. 2012; Kuhn et al. 2013; Trummer et al. 2013).

An interesting study published early in 2014 demonstrated similar results in terms of the ability of the rs12785878 and rs3829251 SNPs in the DHCR7 gene to predict 25(OH)D levels in the IMPROVE study, which comprises European subjects (Strawbridge et al. 2014). Additionally, the authors described the ability of the latter SNP to influence the progression of atherosclerosis, through measuring carotid intima-media thickness, in a type 2 diabetes status-dependent, 25(OH)D-independent manner. Further results were provided by this study in terms of the influence of their studied SNPs on the mRNA levels of genes in the vicinity of the corresponding SNPs. In this regard, it was shown that the 25(OH)D-lowering allele of the rs3829251 SNP is also associated with significantly lower DHCR7 mRNA levels in both the liver and aortic adventitia. If such results were to be extrapolated to the proteome level, and subsequently to reflect the translated protein's activity, this would essentially lead to a very interesting conclusion which would not line conventionally with currently understood norms. In other words, this would mean that although this specific allele of the rs3829251 SNP leads to a possibly reduced expression of DHCR7, and ultimately less flux of 7DHC through the cholesterol biosynthetic pathway and more toward vitamin D3 photosynthesis, this allele is associated with lower 25(OH)D levels. While this study described, for the first time, the relationship between allelic

variations and mRNA expression levels for DHCR7, their investigation was confined to tissues that are not the primary vitamin D3-producing sites, which may present a different association between the observed alleles and DHCR7 mRNA levels.

GC

Thegroup-specific component (GC) was originally identified and named by Hirschfeld in 1959 (Hirschfeld 1959). Several years later, Daiger and colleagues elicited the most recognized, but not the only role the protein plays, transport of vitamin D (Daiger et al. 1975). Besides this widely recognized job, VDBP has been shown to take part in the transport of fatty acids and actin scavenging, which demonstrates potential of use as a novel therapeutic candidate, as demonstrated by recent attempts to develop DBP analogs for therapeutic purposes (Speeckaert et al. 2014).

The majority of vitamin D and its major metabolites circulate the body bound to this protein as well as other albumin proteins. After the first hydroxylating step in the liver, 25(OH)D bound to VDBP undergoes renal activation, which occurs via uptake of the 25(OH)D-VDBP complex via the transmembrane receptor, megalin (Chun et al. 2014). This model supports the importance of VDBP in the renal activation of 25(OH)D, since increased urinary loss of vitamin D metabolites was observed in VDBP knockout mice (Chun et al. 2014). On the other hand, it was recently shown that cells cultured in serum from VDBP knockout mice exhibited increased sensitivity to $1,25(OH)_2D$ compared to control counterparts (Chun et al. 2014).

Interestingly, cells expressing extrarenal 1α-hydroxylating enzyme appear to lack megalin, thus highlighting the possible acquisition of those cells by means other than those involving megalin, perhaps via passive diffusion of free or bioavailable 25 (OH)D, hence the "free hormone hypothesis" (Chun et al. 2014).

Genetically, three alleles of this gene product have been identified through isoelectric focusing, with noticeable variations in frequencies across populations (Speeckaert et al. 2014). The observed alleles originate from two non-synonymous SNPs in exon 11, namely, rs7041 (Gc1) and rs4588 (Gc2) (Speeckaert et al. 2014). Different combinations of the two SNPs lead to the presence of two Gc1 isoforms, Gc1F and Gc1S (Speeckaert et al. 2014). The three alleles have differing affinities to vitamin D, where Gc1F has been shown to possess the highest affinity, then Gc1S, and finally Gc2 (Speeckaert et al. 2014).

In terms of relationship of GC SNPs with disease and 25(OH)D levels, results so far have provided similar conclusions, as with the case of DHCR7 SNPs, where the SNPs have been shown to predict 25(OH)D concentrations in various cohorts, but not disease incidence.

Kühn and coworkers (2013), for instance, investigated two SNPs in the GC gene (rs1155563 and rs2282679) and reported an association between these SNPs and 25 (OH)D levels, but not with MI incidence, stroke, or total CVD. In a complementary study, Jorde and coworkers (2012) genotyped seven SNPs in the GC gene as part of their study, including the two SNPs included in the aforementioned study, as well as the rs7041, which was also shown by GWAS to influence 25(OH)D levels. With the

exception of the rs222020, all of the GC SNPs included in their study predicted 25 (OH)D levels; however, none of them influenced their main cardiovascular end point, MI. Similarly, in a study including the rs2282679 SNP of the GC gene, the authors describe the association of this polymorphism with 25(OH)D levels, but not with cardiovascular mortality (Trummer et al. 2013).

CYP2R1

Like many aspects of vitamin D research, the identity of the 25-hydroxylating enzyme, responsible for production of the circulatory biomarker, remains unclear. It is generally accepted that the enzyme coded for by the CYP2R1 gene is the main, however not exclusive candidate, based on findings of a recently conducted study employing CYP2R1 null mice. Results presented by Zhu et al. (2013) illustrated the presence of significantly lower concentrations of 25(OH)D in CYP2R1 null mice compared to their wild-type counterparts, nonetheless exhibiting detectable levels of 25(OH)D.

Further clinical evidence highlighting CYP2R1's fundamental 25-hydroxyalting role comes from two independent studies involving three subjects, male and female Saudi siblings and a Nigerian male, presenting with a rare genetic mutation in exon 2 of the gene (Cheng et al. 2004). The detected non-synonymous mutation changes leucine at position 99 to proline, which appears to be highly conserved across several species, thus resulting in severe 25(OH)D deficiency. In line with these findings are the results of the GWAS indicating a role of CYP2R1 gene polymorphisms in predicting 25(OH)D levels (Ahn et al. 2010; Wang et al. 2010).

Among the CYP2R1 SNPs that has been highlighted in several publications, including those involving GWAS results, is the rs10741657 SNP. In a European population, this SNP has demonstrated association with circulating 25(OH)D levels, but not with cardiovascular outcomes (Jorde et al. 2012). Surprisingly, in two different European cohorts, the same SNP was not found to predict 25(OH)D levels (Kuhn et al. 2013; Trummer et al. 2013). Interestingly, in our study which involved Egyptian subjects, this SNP predicted both 25(OH)D levels and CAD incidence, where the 25(OH)D-lowering allele was associated with disease incidence, thus highlighting the potential use of this SNP as a genetic marker of CAD (Hassanein et al. 2014). Moreover, the rs2060793 SNP, which was shown to influence 25(OH)D levels by GWAS, was not found to influence MI incidence by Jorde et al. (2012).

CYP27B1

The exploration of mitochondrial 1-hydroxylase, encoded by the CYP27B1 gene, in numerous cells shed light on vitamin D's autocrine mechanism of action. This in part contributed significantly to the recent vitamin D renaissance. Furthermore, the decreased expression of this gene in various cancers highlights its possible crucial role in maintaining therapeutic levels of 1,25(OH)2D in target cells (Jones et al. 2012).

Although SNPs in the gene encoding the 1α-hydroxylase do not predict circulating 25(OH)D levels, according to the conducted GWAS, it is quite comprehendible that common variants in the gene encoding the sole activating enzyme in the vitamin

D metabolic pathway may influence the susceptibility to diseases presumably affected by vitamin D status.

A recently conducted study, aimed to investigate the connection between vitamin D-related genetic variants and obesity traits, included two SNPs in the CYP27B1 gene (rs1048691 and rs10877012) and concluded a lack of association of these SNPs with obesity traits, such as body mass index (BMI) and waist-hip ratio (Vimaleswaran et al. 2013). Similarly, a study conducted on Chinese subjects genotyped seven SNPs in the CYP27B1 gene and demonstrated a lack of association with BMI (Dorjgochoo et al. 2012).

On a similar note, Kuhn et al. (2013) illustrated a lack of triangular relationship between the rs10877012 SNP of the CYP27B1 gene, 25(OH)D levels, and risk of CVD. Putting together results of these three aforementioned studies, it seems that polymorphisms in the CYP27B1 gene may not play a causal role in the development of CVD or associated risk factors, namely, obesity; however, the rs4646536 intronic SNP was found to influence the incidence of congestive heart failure in hypertensive individuals (Wilke et al. 2009).

CYP24A1

CYP24A1 is the enzyme responsible for the catabolism of the major vitamin D metabolites, 25(OH)D and 1,25(OH)2D. Besides this basic biochemical function, the activity of this enzyme has been the focus of diverse research, where studies have demonstrated the increased activity of this enzyme in various cancers, possibly reducing the amounts of calcitriol inside target cells, whereas others have illustrated that inactivating mutations in the gene encoding this protein lead to a disease known as genetically linked idiopathic infantile hypercalcemia, which is characterized by an abolished activity enzyme, and thus, as its name implies, hypercalcemia, possibly resulting from accumulating amounts of vitamin D (Jones et al. 2012).

Pharmacologically, CYP24A1 rises as a promising therapeutic target, based on the current use of vitamin D analogs in the treatment of hyper-proliferative diseases such as psoriasis, which ultimately leads to increased expression of CYP24A1 in target cells with subsequent increase in 1,25(OH)2D degradation (Jones et al. 2012). Additionally, studies have illustrated an increased expression and reduced silencing of the CYP24A1 gene in various cancers, which altogether emphasizes the need for highly specific CYP24A1 inhibitors or chemically modified, catabolism-resistant vitamin D analogs (Jones et al. 2012).

With regards to genetics, SNPs in the CYP24A1 gene have been linked to 25(OH)D status, which fits well with the premise that such mutations may affect the enzyme's activity, and in turn, the metabolite's catabolism and overall concentration in the body. The rs6013897 SNP of the CYP24A1 gene was shown to predict 25(OH)D levels by the GWAS published by Wang et al. (2010) who performed their study on European cohorts. Interestingly, the same SNP was not found to predict 25(OH)D levels by the study conducted by Kuhn et al. (2013) who also performed their study on a European cohort. In the same study, the SNP was not found to predict the incidence of CVD. On the other hand, Jorde et al. (2012) conducted a similar study, also involving European

subjects, and included an additional CYP24A1 SNP, rs2762939, and demonstrated a significant association between rs6013897 and 25(OH)D levels, but a similar observation for the other SNP was not observed. Furthermore, the authors reported a lack of association between the investigated SNPs and MI and type 2 diabetes incidences. Moreover, Shen et al. (2010) reported an association between the rs2762939 SNP and coronary artery calcification, which predicts CVD risk.

VDRG

VDR genetics are arguably the most investigated of all vitamin D-related genes. So far, several genetic polymorphisms, identified by restriction enzymes, have been mapped to both noncoding and coding regions of the VDRG (Uitterlinden et al. 2004). Since the VDR is responsible for exerting calcitriol's actions, it is thinkable that mutations in the corresponding gene may alter the susceptibility to various pathologies. Furthermore, numerous publications on VDR knockout mice have demonstrated the increased potential of such model to develop CVD and cancer (Bouillon et al. 2008), which illustrates the importance of VDR activation in counteracting such diseases.

Among the positive associations of VDRG SNPs with CVD are the ones reported in the study by Ferrarezi et al. (2013), where the investigators highlighted the association of the ApaI, TaqI, and BsmI with CAD risk in type 2 diabetics. On the other hand, the ApaI and TaqI polymorphisms were not found to predict CAD incidence in an Egyptian cohort, whereas the former was found to be associated with 25(OH)D levels (Abu El Maaty et al. 2015).

In a study conducted on diabetic Caribbean patients, the authors described the association of 25(OH)D levels with both the FokI and ApaI polymorphisms (Velayoudom-Cephise et al. 2011). Similarly, Hossein-Nezhad et al. (2014) reported an association of vitamin D deficiency with the FokI polymorphism, as well as the association of the latter with degree of collateralization. Recently, Prabhakar et al. (2015) further elucidated the potential important role the FokI polymorphism may play via demonstrating its gender-specific association with ischemic stroke in an Asian Indian cohort. In a Caucasian cohort, Vaidya et al. (2011) illustrated a connection between the only protein polymorphism found in the VDRG, FokI, and plasma renin activity.

In terms of the BsmI polymorphism of the VDRG, studies have demonstrated its association with CVD biochemical risk factors (Laczmanski et al. 2013), MI (Ortlepp et al. 2005), and left ventricular hypertrophy in patients with chronic kidney disease (Santoro et al. 2014).

On the other hand, studies challenging the association of VDRG SNPs with CVD have also been reported. For example, Pan et al. (2009) reported a lack of association, in a Chinese cohort, between the FokI and BsmI polymorphisms and CAD. Similarly, in a larger study cohort, Ortlepp et al. (2003) showed that the BsmI polymorphism is associated with neither CAD incidence nor severity. In an Indian cohort of patients with verified CAD, five investigated SNPs in the VDRG were not found to be associated with disease incidence (Shanker et al. 2011).

On the Long-Standing Relationship of Vitamin D with CVD

More than three decades ago, precisely in 1981, Robert Scragg published a back then unconventional hypothesis linking seasonal fluctuations of UV radiation (which reflect year-round changes in vitamin D levels) with CVD mortality (Scragg 1981). His hypothesis was built on compelling evidence available at that time, such as associations of latitude, altitude, and age with CVD mortality, all of which have profound connections to vitamin D levels. However, it was only several years later that both molecular and epidemiological evidences were available to support his hypothesis.

In 1989–1990, results of two complementary studies, conducted in institutes thousands of miles apart, significantly contributed to our current awareness of the vitamin D-CVD connection. On one hand, Eberhard Ritz's laboratory in Heidelberg, along with collaborators, managed to publish their findings on the presence of VDRs in endothelial cells in *The Journal of Clinical Investigation* (Merke et al. 1989). Almost a year later, researchers from the University of Auckland published the first report of the association of circulatory 25(OH)D levels with myocardial infarction (MI) in the *International Journal of Epidemiology* (Scragg et al. 1990).

Since then, and with the immense progress in molecular biology techniques, our understanding of the relationship has been reshaped. Vitamin D seems to present profound cardioprotective properties on various aspects of cardiovascular health, most notably endothelial function.

An imbalance in nitric oxide (NO) homeostasis that leads to endothelial dysfunction emerges as a direct therapeutic target of calcitriol. Studies have illustrated an increase in NO production in cultured endothelial cells upon treatment with vitamin D (Molinari et al. 2011), as well as an upregulation of endothelial NO synthase (eNOS) gene (NOS3) expression (Talmor-Barkan et al. 2011), which is the enzyme responsible for NO production, using the amino acid L-arginine as a substrate. In line with these results are those provided by Andrukhova et al. (2014) and Ni et al. (2014). The former illustrated that mice harboring a mutated, nonfunctional VDR, fed a diet aimed to restore calcium homeostasis, exhibited decreased NOS3 mRNA and proteins levels compared to wild-type mice, independent of the renin-angiotensin-aldosterone system (RAAS). Similarly, using a mouse model with selectively knocked out VDR in endothelial cells, Ni et al. (2014) demonstrated decreased eNOS mRNA and protein levels in aortas of their transgenic animal models, compared to wild types. Furthermore, the same group showed an increased susceptibility to the deleterious effects of infused angiotensin II in the same mouse model, in terms of blood pressure measurements, as well as an increase in natriuretic peptide type A and B gene expression, which act as reliable markers for cardiac conditions.

Additionally, Valcheva et al. (2014) reported an increase in cathepsin D mRNA and protein levels in vascular smooth muscle cells obtained from VDR knockout mice compared to controls. This enzyme possesses renin-like effects, consequently increasing local angiotensin II production, which has also been observed in their study, along with an increase in angiotensin II type 1 receptor mRNA and

protein levels in VDR knockout cells compared to wild type. Interestingly, the same paper illustrated an increase in $p57^{Kip2}$ mRNA and protein levels in VDR knockout cells, which has been previously identified as a strong inducer of cellular senescence. Furthermore, Chen et al. (2011) illustrated increased left ventricular weight and myocyte size in transgenic mice with selectively knocked out VDR in myocytes.

On the other hand, the effect of vitamin D on the endogenous inhibitor of eNOS, asymmetric dimethylarginine (ADMA), has been controversial, with conclusions of an inverse association between the two biomarkers, 25(OH)D and ADMA, or a lack of any association (Ngo et al. 2010; Abu El Maaty et al. 2013).

Other beneficial effects of vitamin D on other aspects affecting endothelial function have been published, namely, effects on oxidative stress and inflammation (Abu El Maaty and Gad 2013). Furthermore, calcitriol has been shown to positively affect the RAAS, via a downregulation of renin gene expression (Li 2011). This effect holds a significant advantage over clinically used antihypertensive medication, such as angiotensin-converting enzyme (ACE) inhibitors, which interrupt the homeostatic negative feedback mechanism of the RAAS, orchestrated by angiotensin II type I receptor, resulting in increased levels of renin (Li 2011). Calcitriol, on the other hand, affects the expression of renin and not just impacts the activities of enzymes in the system or blocks the effects of angiotensin II (Li 2011).

Recently, an elaborate study by Dong et al. (2012) showed increased protection against oxidative stress in human renal arteries cultured from hypertensive patients upon treatment with calcitriol, via illustrating a significant reduction in reactive oxygen species (ROS) production, in the presence of angiotensin II, as well as a modulation of expression of involved genes, downregulating those coding for NADPH oxidase subunits, namely, NOX4 and P67PHOX, which are responsible for generating ROS and increasing the expression of superoxide dismutase-1 (SOD-1).

Epidemiologically, low 25(OH)D levels have been associated with various CVDs, as well as risk factors associated, such as type 2 diabetes, hypertension, and hypercholesterolemia (Muscogiuri et al. 2012; Abu El Maaty and Gad 2013). Although results of numerous interventional studies have been published, yielding conflicting results, many of these studies are underpowered by several key points, such as insufficient dosing or treatment of subjects exhibiting normal 25(OH)D levels (Hsia et al. 2007; Pilz et al. 2013), as previously mentioned.

Moreover, vitamin D deficiency leads to an increase in parathyroid hormone (PTH) levels, which in turn leads to inflammation as well as stimulation of the RAAS (Abu El Maaty and Gad 2013).

It is quite clear that the molecular genetic basis of the relationship is the youngest member of a long-standing family of players linking vitamin D with CVD. With more and more discoveries under way, it is quite conceivable that the next coming years would witness a much better understanding of the underlying mechanisms by which vitamin D impacts cardiovascular health, in light of recent reports demonstrating vitamin D's effects on the various signaling pathways involved in differentiation, proliferation, and aging.

Potential Application to Prognosis, Other Diseases, or Conditions

In terms of noncommunicable diseases, CVD remains the number one cause of death globally. Second in line comes cancer. With the immense research done in this area, similar to CVD, vitamin D's role in cancer remains neither well defined nor fully understood. It is well established in various forms of cancer that vitamin D induces apoptosis and differentiation and inhibits proliferation, metastasis, angiogenesis, and inflammation (Feldman et al. 2014). The intricate, complex mechanisms by which these effects are exerted are subject to intense research, with proposed involvement of tumor suppressors such as P53 (Maruyama et al. 2006) and oncogenes like MYC (Feldman et al. 2014). Additionally, introduction of new players, regulatory non-coding RNA, namely, microRNAs (miRs), has opened up new horizons for vitamin D-induced changes in gene expression (Giangreco and Nonn 2013).

In terms of vitamin D genetics, the situation seems quite comparable to that with CVD, with several publications on the effects of vitamin D SNPs on circulatory 25 (OH)D levels; however, investigation into triangular relationships between SNPs, 25 (OH)D levels, and disease remains a fertile ground for research. Additionally, results obtained from studies aimed to link SNPs in the vitamin D pathway with various cancers have been somewhat conflicting (Feldman et al. 2014).

In a study involving breast cancer patients, Reimers et al. (2015) reported the association of several polymorphisms in the CYP24A1 gene that reduce the risk of breast cancer incidence, with the rs6068816 SNP having the highest degree of significance. Moreover, the common polymorphism in the VDRG, TaqI, was also found to reduce breast cancer risk in the same study. Contrastingly, results obtained from the Breast and Prostate Cancer Cohort Consortium (BPC3) revealed a lack of association between vitamin D-related SNPs and risk of breast cancer (Mondul et al. 2015). Similarly, Dorjgochoo et al. (2011) reported a lack of connection between vitamin D-related genetic variants and risk of breast cancer in a cohort of Chinese women.

Similar contradicting results have been obtained for prostate cancer risk in relation to vitamin D-related genetic variants, where a study conducted on Korean males (Oh et al. 2014) as well as the Health Professionals Follow-up Study (Shui et al. 2012) illustrated a significant association between their investigated SNPs and prostate cancer risk, whereas results obtained from the BPC3 support a lack of such association (Mondul et al. 2013).

In the HCT116 colorectal cancer cell line, and using site-directed mutagenesis to introduce five and four non-synonymous substitutions in the CYP27B1 and CYP24A1 genes, respectively, Jacobs et al. (2013) showed significant alterations in enzymatic activities of both translated proteins compared to wild types, essentially resulting in varying biological effects, in terms of VDR activation, assessed using an RXR-VDR mammalian two-hybrid system, as well as RT-qPCR. This ultimately provides molecular evidence on the importance of common genetic variations in the vitamin D pathway.

Summary Points

- Low circulating vitamin D levels are associated with a number of chronic diseases, thereby acting as an independent marker for those diseases.
- A "mega-trial" aimed to determine the efficacy of supplemental vitamin D on cardiovascular outcomes is currently under way.
- GWASs have illustrated that SNPs in vitamin D-related genes can predict 25(OH) D levels in a number of populations.
- Some studies have demonstrated that select SNPs may predict both disease incidence and 25(OH)D levels.
- Calcitriol appears to impact the cardiovascular system, via beneficially affecting the RAAS, minimizing oxidative stress, and restoring normal NO production.

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Nitric Oxide Regulating Proteins as Biochemical and Genetic Markers of Coronary Artery Disease

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_34

Abstract

Cardiovascular disease (CVD) remains the leading cause of death worldwide. Despite huge efforts and great advances in studying the genetic component of CVD, there is still a great need for exploring the genetic and environmental factors contributing to the development of this disease. Among these factors evolve modulation of nitric oxide (NO) homeostasis and oxidative stress as central players according to recent reports. A wide range of biochemical disturbances, including reduced bioavailability of NO and oxidative stress, has been shown to be associated with endothelial dysfunction (ED). Many studies described the contribution of ED in the predisposition of CVD, particularly coronary artery disease (CAD). Recent evidence indicates that ED may be genetically determined. This chapter points out to the key players that influence vascular NO levels and their role in the protection against and/or predisposition to CAD.

Keywords

Nitric oxide • SNP • Nitric oxide synthase • Coronary artery disease • DDAH • Paraoxonase • NADPH oxidase • Endothelin • Ox-LDL

Abbreviations

ADMA	Asymmetric dimethylarginine
AMI	Acute myocardial infarction
ARE	Arylesterase
CABG	Coronary artery bypass grafting
CVD	Cardiovascular disease
DDAH	Dimethylarginine dimethylaminohydrolase
ED	Endothelial dysfunction
EDN-1	Endothelin-1 gene
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
HDL	High-density lipoprotein
hs-CRP	High-sensitivity C-reactive protein
IHD	Ischemic heart disease
iNOS	Inducible nitric oxide synthase
LDL	Low-density lipoprotein
L-NMMA	Levo-N-monomethyl arginine
MI	Myocardial infarction
NADPH	Hydrogenated nicotinamide adenine dinucleotide phosphate
NHI	National Heart Institute
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
O_2^-	Superoxide radical
$ONOO^-$	Peroxynitrite
Ox-LDL	Oxidized low-density lipoprotein

PCI	Percutaneous coronary interventions
PCR	Polymerase chain reaction
PKC	Protein kinase C
PON	Paraoxonase
RFLP	Restriction fragment length polymorphism
ROSs	Reactive oxygen species
SDMA	Symmetric dimethylarginine
sGC	Soluble guanylate cyclase
SNP	Single nucleotide polymorphism
TG	Triacylglycerols
VSMCs	Vascular smooth muscle cells

Key Facts of Nitric Oxide

- Nitric oxide (NO) is the smallest, lightest molecule – and the first gas – known to act as a biological messenger in mammals.
- NO is identical to EDRF (endothelial-derived relaxing factor), well described in literature before the identification of NO.
- NO participates in the control of vascular tone as an antagonist of the adrenergic regulatory system.
- NO prevents atherosclerosis by vascular smooth muscle relaxation as well as inhibiting platelet aggregation, leukocytes migration and adhesion, and vascular smooth muscle proliferation.
- Intracellular NO production, from L-arginine, is catalyzed by several isoforms of an enzyme termed nitric oxide synthase (NOS, EC1.14.13.39)
- Three major NOS isoforms have been identified in humans: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS).
- The most obvious effector pathway for NO is activation of soluble guanylate cyclase (sGC).
- NO has dual roles in the human body; it has several physiological roles in cardiovascular, respiratory, GIT, and reproductive systems and in immunity, while overproduction of NO is incriminated in the pathogenesis of several conditions such as septic shock, epilepsy, tissue damage, inflammation, and nerve damage left by the stroke.
- NO reacts with reactive oxygen species to form toxic peroxynitrite anions ($ONOO^-$).
- Therapeutic strategies related to NO involve both increasing and decreasing NO.

Definitions

Acute A disease with a rapid onset and/or a short course, mostly presented in severe form. However, not all acute diseases or injuries are severe.

Allele One of a number of alternative forms of the same gene may be reflected in a different phenotype.

Asymmetric dimethylarginine (ADMA) Endogenous physiological inhibitor of nitric oxide synthase.

Cardiovascular disease A class of diseases involving the heart and blood vessels.

Coronary artery bypass grafting (CABG) Also known as heart bypass. It involves "open" heart operation which bypass stenotic arteries by grafting vessels from elsewhere in the body.

Also known as ischaemic heart disease (IHD) and coronary heart disease (CHD). It is a group of disease that includes stable angina, unstable angina, and myocardial infarction.

Genotype Genetic makeup of a cell, an organism, or an individual.

Genotyping Process of assessing the differences in genetic makeup by examining DNA sequence.

Myocardial infarction (MI) Death of heart cells as a result of coronary ischemia

A free radical. It acts in mammals, including human, as an important cellular signaling molecule involved in many physiological and pathological processes.

Nitric oxide synthase (NOS) Enzyme that uses L-arginine and molecular oxygen as substrates to produce NO and the amino acid L-citrulline.

Percutaneous coronary intervention (PCI) Commonly known as coronary angioplasty. It is a nonsurgical procedure used to treat narrowed coronary arteries by inserting a stent or deflated balloon to open the artery.

Polymorphism The occurrence of more than one form in the same population or species.

Single nucleotide polymorphism (SNP) A variation between individuals of a specific population at a single nucleotide in their DNA. By definition, it occurs at above 1 % of the population.

Introduction: Key Players in the Control of Vascular NO Levels

In the blood vessels, NO is produced from the endothelium mainly by the constitutively expressed endothelial nitric oxide synthase (eNOS), which is activated by shear stress of the flowing blood or agonists such as bradykinin and acetylcholine.

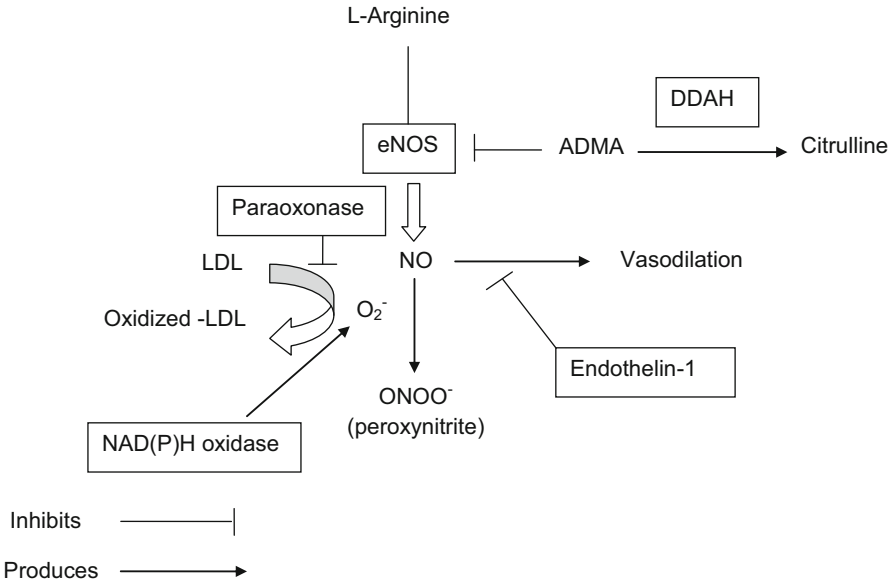

Fig. 1 Enzymes and proteins that control vascular NO levels. This figure comprises several enzymes and proteins that affect NO homeostasis including, asymmetric dimethylarginine (ADMA), oxidized LDL, paraoxonase activity, NAD(P)H oxidase activity, and dimethylarginine dimethylaminohydrolase (DDAH) activity. Dimethylarginine dimethylaminohydrolase (DDAH)

Besides its role as relaxing factor, NO protects blood vessels from thrombosis, by inhibiting platelet aggregation and adhesion. In addition, endothelial NO possesses multiple anti-atherosclerotic properties, which include (I) prevention of leukocyte adhesion to vascular endothelium and leukocyte migration into the vascular wall; (II) decreased endothelial permeability, reduced influx of lipoproteins into the vascular wall, and inhibition of low-density lipoprotein (LDL) oxidation; and (III) inhibition of DNA synthesis, mitogenesis, and proliferation of vascular smooth muscle cells. Reduced bioavailability of eNOS-derived NO or reduction of its biosynthesis markedly contributes to atherogenesis and thereby to MI (Jones and Hingorani 2005). The interrelationship and regulatory mechanisms that control vascular NO levels are quite complex. Several biochemical parameters and enzymes that contribute in these mechanisms are shown in Fig. 1.

In the early 1990s, an endogenous inhibitor to the nitric oxide synthase (NOS) pathway has been identified, namely, asymmetric dimethylarginine (ADMA). Accumulation of ADMA in the plasma of patients in several diseases, including CVD, reduces the release of endothelium-derived NO (Vallance et al. 1992a).

After uptake from the circulation, ADMA is degraded mainly by an intracellular enzyme termed dimethylarginine dimethylaminohydrolase (DDAH). DDAH degrades ADMA to citrulline and dimethylamine. Two isoforms of DDAH have been identified, DDAH-1 and DDAH-2, which regulate to a great extent the level of

ADMA in the blood and tissues. Thus, DDAH, through catabolism of ADMA, regulates the activity of NOS. Consequently, DDAH dysfunction may be a crucial unifying feature of increased cardiovascular risk. Lieper et al. have shown that loss of DDAH-1 activity leads to accumulation of ADMA and reduction in NO signaling (Leiper et al. 2007). This in turn causes vascular pathophysiology, including endothelial dysfunction, increased systemic vascular resistance, and elevated systemic and pulmonary blood pressure.

The serum high-density lipoprotein (HDL) concentration is inversely correlated with risk of MI. The mechanism for this continues to be the subject of considerable debate. High-density lipoproteins are thought to protect LDL from oxidation due to the presence of antioxidant enzymes, among which paraoxonase (PON) [EC.3.1.8.1, aryldiakylphosphatase] seems to be of major importance (Durrington et al. 2001). Paraoxonase represents an endogenous defense mechanism against vascular oxidative stress, thereby contributing to the prevention of atherosclerosis (Horke et al. 2007).

Oxidative stress in the vasculature induced by superoxide anion has been implicated in the pathogenesis of coronary artery disease (CAD). In two studies from our lab, it has been demonstrated in the first evidence that oxidative stress and ADMA are associated with cardiovascular complications in hemodialysis patients (El-Mesallamy et al. 2008), whereas it has been provided in the second evidence that free radicals are implicated in the development of atherosclerosis induced by hypercholesterolemia (Gad et al. 2014).

The sources of superoxide production in the vasculature are diverse and include vascular smooth muscle cells (VSMCs), endothelial cells, and macrophages. Although NADPH oxidase enzyme was originally described in phagocytes, it has recently become evident that the NADH/NADPH oxidase system is an important enzymatic origin of superoxide radical in nonphagocytic cells such as VSMCs and endothelial cells. NADPH oxidase is a major cause of atherosclerosis, and NADPH oxidase inhibitors may reverse atherosclerosis. NADPH oxidase produces reactive oxygen species (ROSs). These ROSs activate an enzyme that makes the macrophages adhere to the artery wall. This process is counterbalanced by NADPH oxidase inhibitors and by antioxidants. It is postulated that atherosclerosis is primarily mediated through the oxidation of LDL (Park et al. 2009).

Vascular tone is regulated by vasodilators and vasoconstrictors. Endothelin-1 (ET-1) is the predominant vasoconstrictor peptide that constricts vascular smooth muscle, whereas NO is the primary vasodilator peptide that relaxes vascular smooth muscle. Kurita et al. inferred in their study the importance of plasma NO/ET-1 ratio as a useful biological marker for predicting CAD (Kurita et al. 2005). High levels of ET-1 impair endothelial NO production via an isoform-specific PKC-mediated inhibition of eNOS expression (Ramzy et al. 2006). Thus, the endothelin system plays a central role in the control of myocardial function and its pathophysiology.

NO Key Players and CAD

Myocardial infarction (MI) is a complex multifactorial and polygenic disorder which is thought to result from an interaction between a person's genetic makeup and various environmental factors. Conventional risk factors for MI include hypertension, diabetes mellitus, and hypercholesterolemia. Although each risk factor is partly under genetic control, a family history of MI is also an independent predictor, suggesting the existence of additional susceptibility genes for this condition.

Furthermore, some patients who have suffered a MI do not have any conventional risk factors, suggesting the contribution of an uncharacterized genetic component. Genetic-linkage studies and candidate-gene analyses have implicated several candidate genes in the predisposition to MI. Among the genetic variants known to increase the risk of MI are those of angiotensin-converting enzyme, platelet aggregation IIIa, coagulation factor VII, and cholesteryl ester transfer protein (Yamada et al. 2002). Few studies were done on enzymes and regulatory proteins that control vascular NO metabolism.

The eNOS/NO Arm

Genetic Variation in the eNOS Gene

To date more than 100 polymorphisms have been identified in, or in the vicinity of, the eNOS gene (NCBI SNP database, http://www.ncbi.nlm.nih.gov/SNP/). Among them, 15 polymorphisms exist in the eNOS promoter that might influence mRNA transcription and reduce gene expression (Jones and Hingorani 2005). However, the Glu298Asp (rs1799983) polymorphism in exon 7 was shown to be the only common variation that leads to amino acid substitution in the mature protein (Hingorani et al. 1999). In this polymorphism the guanine at position 894 is substituted by thymine, leading to a change in the amino acid at position 298 from glutamate to aspartate.

A meta-analysis of the Glu298Asp polymorphism in 19 different population study (9252 subjects) reported that the wild-type GG is the predominant genotype representing 67 %, while the GT and TT genotypes are present in 28 % and 4 % of the subjects, respectively (Zintzaras et al. 2006). In the Egyptians, it was found that the wild-type GG genotype is prevalent in 58.4 % of the healthy controls, while GT and TT are present in 33.7 % and 7.9 %, respectively (Gad et al. 2012). The allele frequencies of the G and T alleles were 75.3 % and 24.7 %, respectively. No significant differences in the eNOS genotype distribution pattern (Mann-Whitney test, $p = 0.12$) or in the allele frequencies (Mann-Whitney test, $p = 0.09$) between female and male subjects were observed. An earlier study conducted using only ten healthy Egyptian subjects showed genotype frequencies of GG (50 %), GT (40 %), and TT (10 %) (Nagib El-Kilany et al. 2004).

Results of the two Egyptian studies are generally comparable to a study on healthy Caucasians ($n = 171$), which showed that GG is the genotype found in

highest frequency (50.3 %), GT frequency was 39.6 %, and TT was 8.2 % (Walch et al. 2008). Analogous genotype distributions were also demonstrated in other studies for populations of European origin: Germany ($n = 190$; GG 50.5 %, GT 40 %, and TT 9.5 %) (Krex et al. 2006), Turkish ($n = 150$; GG 49.3 %, GT 41.3 %, and TT 9.3 %) (Afrasyap and Ozturk 2004), English ($n = 331$; GG 47.8 %, GT 42 %, and TT 10.2 %) (Hingorani et al. 1999), and in the European HapMap-CEU study ($n = 120$; GG 40.0 %, GT 51.7 %, and TT 8.3 %). The allele frequencies in all these studies ranged from 65.8 % to 71.1 % for the G allele and from 29.0 % to 34.2 % for the T allele.

A remarkably different genotype distribution appeared in Asians where the wild-type GG predominates in around 75 % of the population, while the homozygous Asp variant (TT genotype) is nearly absent. Representative examples are studies from Japan ($n = 513$; GG 84.4 %, GT 17.4 %, and TT 0 %) (Kato et al. 1999) and Korea ($n = 411$; GG 97.6 %, GT 19.5 %, and TT 0.9 %) (Moon et al. 2002). A similar pattern was seen in African American ($n = 60$; GG 70.4 %, GT 23.9 %, and TT 5.6 %) (Li et al. 2004).

Controversial results were reported in the literature with regard to the influence of eNOS Glu298Asp polymorphism on the incidence of CAD. While several studies did not provide enough evidence that this polymorphism influences the risk for CAD in Egyptian, Turkish, and British subjects (Jeerooburkhan et al. 2001; Aras et al. 2002; Gad et al. 2012), others observed an association of the Glu298Asp with the risk of MI in British and Japanese subjects (Shimasaki et al. 1998; Hingorani et al. 1999). These findings further support the previously reported role of ethnicity in determining the prevalence of genetic polymorphisms and their subsequent putative impacts in a given population.

Serum NO Levels

Interest in the measurement of serum NO concentration is increasing since it has been reported that NO levels are influenced by several diseases, including diabetes, heart failure, sepsis, and liver cirrhosis; however, little is known about the normal range and the physiological changes of serum NO concentrations in healthy population.

Comparable average serum levels of NO were seen in Egyptians (30.3 μM) (Gad et al. 2012) and Turkish subjects (32.6 μM) (Afrasyap and Ozturk 2004). A large study utilizing 1983 healthy Iranian subjects showed that the mean serum NO was 24.4 μM (Ghasemi et al. 2008). The mean serum NO was 55 μM in Japanese (Higashino et al. 2007) and 53.11 μM in Korean individuals (Moon et al. 2002). In African Americans, the mean serum NO concentration was reported to be 8.8 μM in subjects with dominant eNOS genotype (GG) and 9.9 μM in subjects with recessive eNOS genotypes (GT + TT) (Li et al. 2004). A comparison between the mean serum NO concentrations of healthy Egyptian female versus male subjects revealed a nonsignificant difference (Gad et al. 2012). Also, no statistical significance was detected when comparing serum NO concentrations of the different age groups (<20, 21–30, 31–40, and >40 years old) and comparing the serum NO concentrations among different Glu298Asp genotypes.

A highly significant increase in the serum levels of NO has been observed in the MI patients (Bermudez Pirela et al. 2000; Gad et al. 2012). The reason for this finding may be attributed to the fact that MI results in an increased myocardial inducible nitric oxide synthase (iNOS) expression and NO production and higher nitrotyrosine levels, leading to myocardial dysfunction and increased mortality (Feng et al. 2001). There was no association between eNOS genotypes and the serum levels of NO in the MI patients of Egyptian and South Indian population (Angeline et al. 2010; Gad et al. 2012). Sanchez et al. concluded from their study of 49 Spanish MI patients that neutrophils from patients during MI showed an increased production of NO and a marked expression of the iNOS isoform (Sanchez De Miguel et al. 2002).

The DDAH/ADMA Arm

Genetic Variation in the DDAH-2 Gene

Genes for DDAH-1 and DDAH-2 are located on chromosomes 1p22 and 6p21.3, respectively (Tran et al. 2000). They are differentially regulated through development (Redel et al. 2015). It is apparent from gene-silencing studies in rats that DDAH-1 plays an important role in regulating serum ADMA levels, whereas DDAH-2 appears to control NO-mediated functions of the endothelium. The DDAH-2 isoform predominates in tissues expressing eNOS, such as the endothelium (Jones and Hingorani 2005). Thus, DDAH, through catabolism of ADMA, regulates the activity of NOS. Few studies have focused on the possibility that the DDAH gene polymorphisms may contribute to the inheritable risk for CVD in humans. No one has identified specific differences among ethnic groups.

The discovery of a functional polymorphism within DDAH2 gene that might promote individual differences in the ability to metabolize ADMA in vivo and, in turn, underlies susceptibility to CVD has been previously addressed by Jones et al. (2003). In this study, the researchers identified several DDAH2 gene polymorphisms, two of them are the subjects of a previous study from our lab: SNP1 (−1151 C/A) present in the promoter region upstream of the noncoding exon 1 and SNP2 (−499 C/G) localized within intron 2 of the gene (Gad et al. 2011).

O'Dwyer et al. (2006) observed that carriage of a G allele at position −449 in the promoter region of DDAH2 gene is associated with increased ADMA levels, which suggests that the DDAH2 gene expression with a G allele of this position is lower than that with a C allele. Maas et al. (2009) have indicated that −1151 A/C (SNP1) and −449 G/C (SNP2) polymorphisms in the DDAH2 promoter region are associated with an increased prevalence of hypertension.

In the Egyptian study, evidence has been provided that DDAH2 (−1151 C/A) or (−499 C/G) polymorphisms are associated with increased risk of early MI (Gad et al. 2011). It was also revealed that DDAH2 SNP1 (−1151 A/C) and SNP2 (−449 G/C) are in complete linkage disequilibrium. An interesting finding in this study is the difference in frequency of DDAH2 SNP1/SNP2 polymorphisms for the studied sample of Egyptians from those reported for other populations. This finding

addresses the inquiry about the evolutionary course of this gene polymorphism among Egyptians.

Data that belong to the HapMap project [http://www.hapmap.org/] infer that for DDAH2 SNP1 (−1151 C/A, rs805304), Europeans (Utah residents with northern and western European ancestry) have the lowest CC variant (8.3 %), as compared to CA (46.7 %) and AA (45 %) variants. Asian (Han Chinese in Beijing, P. R. China) had 13.3 % CC, 55.6 % CA, and 31.1 % AA. Sub-Saharan African (Yoruban in Ibadan, Nigeria) had 78 % CC, 18.6 % CA, and 3.4 % AA. The results of the Egyptian study indicated that the genotype distribution of SNP1 control subjects was 28 % CC, 54 % CA, and 18 % AA (Gad et al. 2011), which is somewhat different from Europeans as well as sub-Saharan African figures. It seems from an evolutionary point of view that geographical distribution affects DDAH2 SNP1 genotype pattern: the farther you go from Africa, the lower frequency of CC and higher AA genotype are manifested. Not surprisingly, the same conclusion applies to DDAH2 SNP2 (−449 C/G, rs805305) site that has polymorphisms strongly associated with those of SNP1. Similar data were displayed for SNP1 and SNP2 polymorphisms in the HapMap project.

An interesting part of the aforementioned Egyptian study is the association of DDAH2 polymorphisms with the severity of coronary insufficiency (unpublished data). In this study, CAD patients were subclassified according to severity of coronary insufficiency, as verified by coronary angiography, into (a) patients under conservative medical treatment (Med, $n = 12$), (b) patients directed for percutaneous coronary interventions (PCI, $n = 41$), (c) patients advised to do coronary artery bypass grafting operation (CABG, $n = 36$), and (d) patients suffering from acute myocardial infarction (AMI, $n = 11$).

Results shown in Fig. 2 demonstrate a noticeable increase in AA/GG (SNP1/SNP2) genotype frequencies moving from the least (controls and Med) to the most severe (CABG and AMI) coronary insufficiency. AA/GG frequencies in CABG and AMI were more than twofolds (38.95 % and 36.4 %) higher than the control values (18 %). The same trend was applied to allele distribution (Fig. 3). An increase in A/G (SNP1/SNP2) was observed moving from controls (45 %) to CABG and AMI (58.3 % and 68.2 %).

No significant correlation was perceived between the serum levels of ADMA, SDMA, L-arginine, and hs-CRP and carriage of specific DDAH2 allele or genotype. A trend of higher, but not significant, ADMA, SDMA, creatinine, and hsCRP and lower L-arginine and L-arginine/ADMA was observed in the AA/GG group as compared to the other two genotypes (Gad et al. 2010).

Serum ADMA and SDMA Levels

ADMA is one among three methylarginines physiologically found in all human tissues and biological fluids. The other two are N-monomethylarginine (L-NMMA) and symmetric dimethylarginine (SDMA) (Fig. 4). Methylarginines are generated by the posttranslational methylation of arginine residues in proteins. Following proteolysis, free methylarginines are released into the cytosol where they accumulate before being removed to the plasma and cleared into the urine by the kidney (Tran et al. 2003). A study by Murray-Rust et al. (2001) established that DDAH

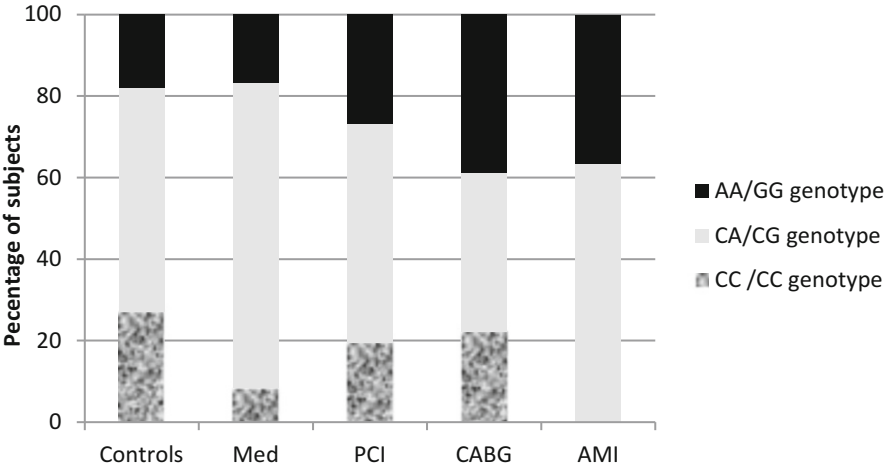

Fig. 2 DDAH2 SNP1/SNP2 genotype distribution in CVD groups as compared to controls. SNP1 = (−1151 C/A, rs805304) and SNP2 (−449 C/G, rs805305). A significant increase in AA/GG genotypes of DDAH2 SNP1/SNP2s, respectively, was observed moving from the controls and med (patients under medication) to the most severe coronary artery bypass grafting (CABG) and acute myocardial infarction (AMI) coronary insufficiency

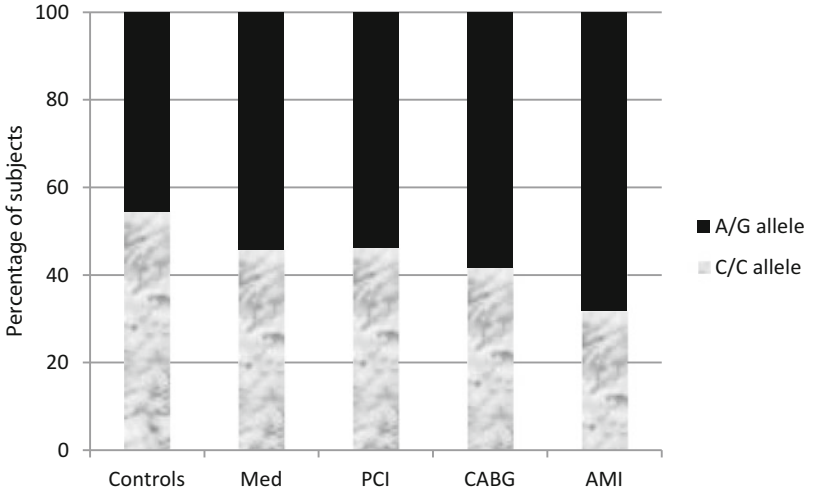

Fig. 3 DDAH2 SNP1/SNP2 allele distribution in CVD groups as compared to controls. SNP1 = (−1151 C/A, rs805304) and SNP2 (−449 C/G, rs805305). A significant increase in A/G alleles of DDAH2 SNP1/SNP2s, respectively, was observed moving from the controls and med (patients under medication) to the most severe coronary artery bypass grafting (CABG) and acute myocardial infarction (AMI) coronary insufficiency

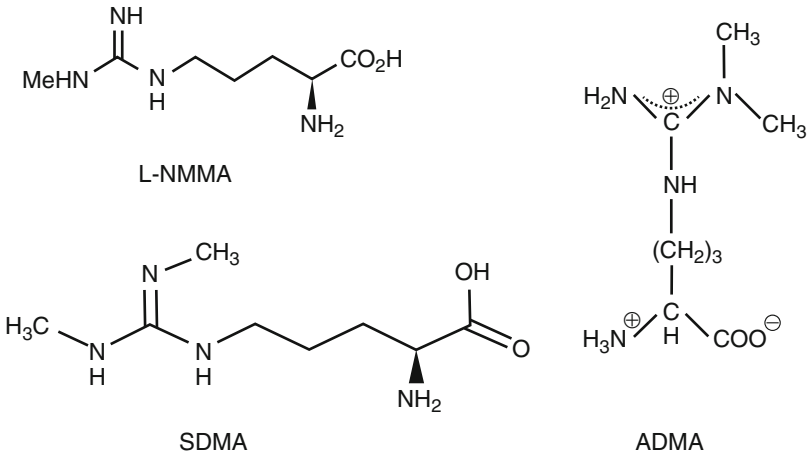

Fig. 4 Methylated arginine identified in eukaryotes. Different forms of methylated arginine physiologically identified in eukaryotes, including the human body. *L-NMMA*, levo-N-monomethylarginine; *ADMA*, asymmetric dimethylarginine; *SDMA*, symmetric dimethylarginine

metabolizes ADMA intracellularly, whereas SDMA is not a substrate for DDAH. Thus, serum ADMA will be dependent primarily on factors that affect DDAH expression and activity, whereas serum SDMA will depend on the rate of renal excretion (Palm et al. 2007). SDMA accumulates to a greater degree (eightfold increase) and more closely parallels creatinine concentration than ADMA. In contrast to ADMA, SDMA does not act as an inhibitor of NO synthase (Vallance et al. 1992b).

Despite the fact that several factors affect the amount of ADMA in tissues, and consequently in the blood, including oxidative stress, hypercholesterolemia, renal function, and DDAH activity, evidence has emerged that ADMA might be a novel cardiovascular risk factor (Boger 2003). However, no significant difference between serum levels of ADMA in controls and CAD patients was observed in the studies of Gad et al. (2010) and Wang et al. (2006) who showed that there was no significant difference in plasma ADMA levels between patients with triple vessel disease and subjects with no detectable coronary disease. Also, levels of SDMA, L-arginine, and L-arginine/ADMA did not differ. In contrast, levels of SDMA in the Egyptian study were surprisingly higher in the CAD patients than controls (Gad et al. 2010).

The ROS/Antioxidants Arm

Genetic Variation in the NADPH Oxidase Gene

All cell types within the heart, including cardiomyocytes, endothelial cells, vascular smooth muscle cells (VSMCs), fibroblasts, and infiltrating inflammatory cells, generate ROS. Potential sources of ROS in these cell types include the mitochondrial electron transport chain, xanthine oxidases, “uncoupled” nitric NOSs, cytochrome

P450, and NADPH oxidase. Among these sources, the NADPH oxidase may be considered unique in that they generate ROS in a highly regulated manner whereas ROSs are generated as by-products of enzymatic activity for all the other sources (Wang et al. 2006).

In the last decade, five NADPH oxidase isoforms each encoded by a separate gene and with distinct tissue distribution have been identified. These isoforms are distinguished by the presence of distinct catalytic subunits, Nox1–Nox5, which mediate the electron transfer process. In addition to the core catalytic Nox subunit, the enzymatic activity of the oxidase depends on additional subunits, which vary according to the isoform. These subunits include gp91phox and p22phox and a cytosolic component composed of subunits p47phox, p40phox, p67phox, and a G protein, Rac (Lassegue and Clempus 2003). In vessels from patients with CAD, expression of Nox2 and Nox4 is enhanced (Guzik et al. 2006). During restenosis of the carotid artery after balloon injury, Nox1, Nox2, and Nox4 are upregulated sequentially at 3, 7–15, and >15 days after injury, respectively (Szocs et al. 2002).

The p22phox subunit is essential to this enzyme's activity, and activation of NADPH through this membrane-bound subunit protein has been shown in vascular cells. Furthermore, many of the stimuli found to activate NADPH oxidase increase expression of the p22phox subunit. The p22phox protein is encoded by the cytochrome b-245, a (CYBA) gene. The CYBA gene is located on the long arm of chromosome 16 (at q24), encodes the alpha subunit of the membrane-bound component, spans 8.5 kilo base (kb), and contains five introns and six exons.

Several CYBA gene variants have been associated with CVD. The C242T (rs4673) CYBA polymorphism has been previously found to influence NADPH oxidase gene expression. This CYBA C242T gene variant is in exon 4 and causes a structural modification in the protein from the histidine-to-tyrosine substitution at residue 72 in a heme-binding site. The resulting structural change in p22phox from this C242T polymorphism has been related to CVD, hypertension, and endothelial function (Feairheller et al. 2009).

Online, Hashad et al. reported an association of a C242T polymorphism of NADPH oxidase p22phox gene with the incidence of AMI (Hashad et al. 2014). The genotype CC in AMI patients was higher by 45 % than controls. This increase was associated with a corresponding rise in ox-LDL (Fig. 5). The study concluded that the wild genotype CC is considered a risk factor of MI and C242T polymorphism of p22phox gene of NADPH oxidase is a novel genetic marker associated with reduced susceptibility to AMI.

Similar results were shown in Asian (Inoue et al. 1998; He et al. 2007) and Finnish populations (Fan et al. 2006). In harmony, Schachinger et al. (2001) observed a significant increase in the flow-dependent dilation in patients bearing the T allele of the C242T polymorphism and an impaired coronary arterial dilator response to nitroglycerin in patients carrying the CC genotype.

Serum Oxidized Low-Density Lipoprotein (ox-LDL) Levels

In 1989, Steinberg et al. (1989) put forward the original oxidative modification hypothesis based on the notion that oxidation represents a biologic modification

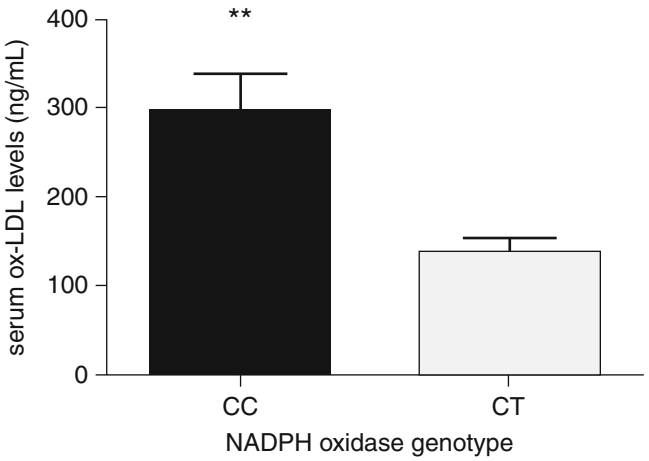

Fig. 5 Correlation between NADPH oxidase genotype distribution and serum levels of ox-LDL in MI patients. TT distribution in MI patients was 0 %. Levels of oxidized LDL were highly elevated in the CC genetic variant of C242T polymorphism of NADPH oxidase p22phox gene in myocardial infarction patients

analogous to chemical modification discovered by Goldstein et al. (1979) that gives rise to foam cells. Since then, numerous studies have supported the ox-LDL hypothesis which says ox-LDL can promote foam cell formation through the so-called "scavenger receptor" pathway. The current oxidative modification or stress hypothesis of atherosclerosis predicts that LDL oxidation is an early, essential event in atherosclerosis that leads to MI and that ox-LDL does contribute to both initiation and progression of atherosclerosis and CAD. Increased levels of ox-LDL have been demonstrated in patients with AMI and unstable angina (Ehara et al. 2001).

Experimental studies have identified several mechanisms through which ox-LDL may contribute to the development of atherosclerosis. Oxidized LDL may cause intimal inflammation by activating expression of adhesion molecules on endothelial cells, stimulating leukocyte chemotaxis, and by inducing release of growth factors from macrophages. A substantial body of evidence suggests that most, if not all, of the atherogenic effects of ox-LDL are derived from the oxidized lipid components. The "active" lipids include both esterified and unesterified peroxidized lipids, lysophosphatidylcholine, cholesterol oxidation products, aldehydes derived from breakdown of both esterified and unesterified oxidized fatty acids, and perhaps proteolipids that may have peroxidized lipids bound to fragmented apoB-10 (Young and Parthasarathy 1994).

Many studies coincide with the above conclusion (Holvoet et al. 1998, 2001; Hashad et al. 2014; Ehara et al. 2001; Fredrikson et al. 2003) suggesting that ox-LDL plays an important role in the progression of CAD and AMI. The question arises regarding what causes high levels of ox-LDL in AMI patients. Are systemic changes involved that alter the lipid profile or is it the atherosclerotic process itself that could be held responsible? At this stage, it is fair to state that this remains speculative.

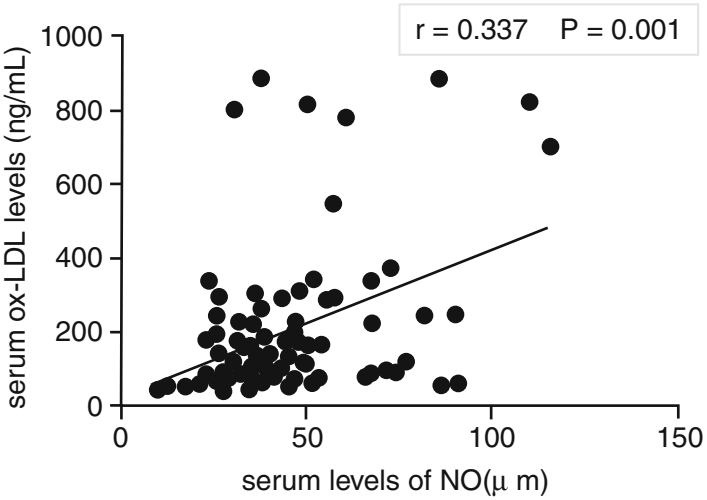

Fig. 6 Correlation between serum NO levels and serum ox-LDL levels in AMI patients. Levels of ox-LDL in the AMI patients are well correlated with serum levels of NO

Previous in vitro studies have documented that macrophages and lymphocytes are capable of oxidizing LDL (Ehara et al. 2001).

It was found in Hashad et al. study (Hashad et al. 2014) that high levels of ox-LDL in the AMI patients are well correlated with serum levels of NO (Fig. 6). NO reacts with O_2^- anion to form ONOO−, a potent oxidant (Beckman and Koppenol 1996). Therefore, NO plays a prooxidant role when present simultaneously with O_2^- anion, which is implicated in the mechanisms of LDL oxidation. Endothelial cells, smooth muscle cells, and macrophages generate O_2^- anion, and thereby ONOO− or other reactive nitrogen intermediates could be formed in the artery wall and lead in part to cell-mediated LDL oxidation (Yoshida and Kisugi 2010).

Genetic Variation in the PON-1 Gene

The serum HDL concentration is inversely correlated with risk of AMI (Durrington et al. 2001). HDL is thought to protect LDL from oxidation due to the presence of antioxidant enzymes, among which paraoxonase (PON) seems to be of major importance (Horke et al. 2007). Paraoxonase represents an endogenous defense mechanism against vascular oxidative stress, thereby contributing to the prevention of atherosclerosis.

The PON gene family in mammals includes at least three members: PON1, PON2, and PON3 (Gupta et al. 2009). The three PON genes share about 65 % similarity at the amino acid level and are located adjacent to each other on chromosome 7 (7q21.3) in humans. Both PON2 and PON3 possess antioxidant properties and lactonase activity, but unlike PON1, they lack the paraoxon or phenyl acetate-hydrolysing activity. PON1 is synthesized in the liver and is closely associated with

Table 1 The genotype distributions and allele frequencies of PON1 Q192R in AMI and control groups. A significant difference was observed in both PON1 genotype distribution patterns ($p = 0.0001$) and the allele frequencies ($p = 0.0002$) between the AMI patients and the controls. The number of subjects is shown in brackets. Patients were randomly employed for the study from the intensive care unit of the National Heart Institute, Imbaba, Egypt. Patients were included if they had a diagnosis of an acute single- or multivessel CAD verified by clinical presentation, ECG changes, and/or cardiac marker elevation. The AMI patients (age range 35 and 55 years) were comprised of 32 females and 52 males. Controls were age and sex matched

	QQ (%)	QR (%)	RR (%)	*p* value
AMI patients ($n = 84$)	34 (40.5 %)	40 (47.6 %)	10 (11.9 %)	0.0001
Control subjects ($n = 100$)	71 (71.0 %)	21 (21.0 %)	8 (8.0 %)	
	Q (%)		**R** (%)	
AMI patients ($n = 84$)	108 (64.3 %)		60 (35.7 %)	0.0002
Control subjects ($n = 100$)	163 (81.5 %)		37 (18.5 %)	

HDL. This most likely explains its ability to metabolize lipid peroxides and to protect against their accumulation on LDL (Durrington et al. 2001).

Several polymorphisms have been reported in the PON1 structure, including Q192 R polymorphism. In this polymorphism the adenine at position 575 is substituted by guanine, leading to a change in the amino acid at position 192 from glutamine to arginine. Numerous studies have been conducted to assess the effect of the PON1Q/R192 polymorphism on susceptibility to CAD. While some studies reported people with the PON1 192 R alloenzyme are more prone to CAD than are those with the Q alloenzyme, others reported no association between PON1Q/R192 polymorphism and CAD (Ombres et al. 1998).

PON1 has esterase and more specifically paraoxonase activity. It was postulated that a single serum enzyme, with both paraoxonase and arylesterase activity, exists in two different isozymic forms with qualitatively different properties and that paraoxon is a "discriminating" substrate (having a polymorphic distribution of activity) and phenylacetate is a "nondiscriminating" substrate for the two isozymes The average activities of serum of individuals of a specific PON1 (Q192) genotype showed higher arylesterase and lower paraoxonase activity than the PON1 (R192) genotype.

The results displayed in Table 1 (unpublished data) showed that the genotype distribution of PON1 gene was significantly different between AMI patients and controls. The corresponding allele frequencies were also significantly different. The odds ratio between QQ genotype and QR+RR genotypes was 3.231 ($p < 0.001$), while the odd ratio between the Q allele and the R allele was 2.256 ($p = 0.001$). The genotypes QR and RR showed higher risk of AMI compared to the homozygous QQ (odds ratio = 3.231, $p < 0.001$). Average PON/ARE ratio showed a significant difference between different genotypes in both AMI patients (QQ 0.91 ± 0.11, QR 1.09 ± 0.11, and RR 2.65 ± 0.4) ($p = 0.0002$) and controls (QQ 0.68 ± 0.1, QR 1.07 ± 0.11, and RR 4.89 ± 2.84) ($p < 0.0001$).

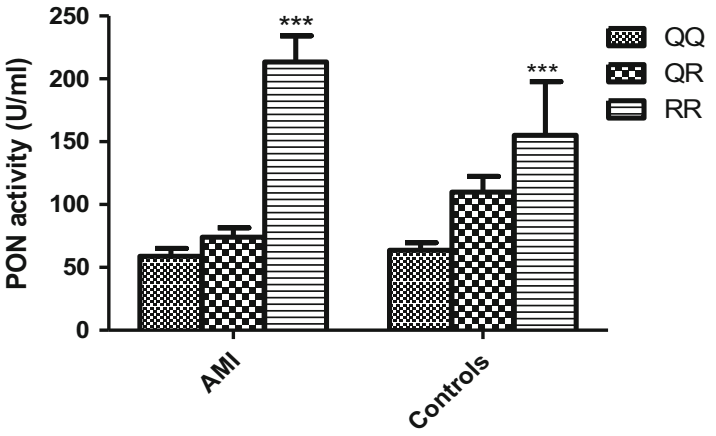

Fig. 7 PON activities among different genotypes in MI patients and controls. Results are expressed as mean ± SEM. ***: Significant difference among various PON1 genotypes at $p < 0.001$. Serum PON activities are significantly different among various PON genotypes in both AMI patients ($p = 0.0009$) and control subjects ($p < 0.0001$) where PON activity is highest in RR genotype then QR genotype and lowest in QQ genotype. Description of subjects is shown in Table 1 legend

Serum PON Activity

PON1 polymorphisms are important in determining the capacity of HDL to protect LDL against oxidative modification in vitro, which may explain the relationship between the PON1 alleles and CAD in case-control studies (Mackness et al. 2001). However, it was suggested that the PON1 phenotype (enzyme activity) is a better predictor of vascular disease than PON1 genotype (Jarvik et al. 2000).

The results displayed in Fig. 7 showed a significant difference in the PON activities among the different genotypes in both AMI patients and control subjects. Similarly, PON/ARE ratios showed a significant difference between different genotypes in both AMI and control subjects. Meanwhile, no significant difference was observed in the ARE activities.

Serum hs-CRP Levels

Inflammation has been proposed to contribute to different stages in the pathogenesis of CHD, including the lifelong process of atherogenesis; the acute atherothrombotic event, which causes ischemic necrosis in AMI; and the myocardial damage following ischemia. Accumulation, aggregation, and oxidative modification of LDL are believed to play an important role in the activation of this inflammation (Entman et al. 1991; Lowe and Pepys 2006).

CRP is the most extensively studied systemic marker of inflammation. CRP is a non-glycosylated circulating plasma protein, which together with the distinct but closely related protein, serum amyloid P component, comprise the pentraxin family of proteins (Pepys and Baltz 1983). CRP is an acute phase reactant that responds as a

sensitive, though nonspecific, marker of systemic inflammation. The pentameric globular protein is synthesized by the liver in response to stimuli from circulating inflammatory cytokines. CRP has traditionally been used as a systemic marker of infection and tissue injury. An expanding body of research now indicates that CRP likely plays a direct, active inflammatory role in blood vessels, leading to the development of atherosclerosis (Szmitko et al. 2003).

Despite many claims and assertions in the literature, neither the normal functions of human CRP nor its possible role in disease is known. This is because neither deficiency or even structural polymorphism of human CRP has yet been reported nor is any drug or other therapeutic maneuvering yet available which specifically inhibits or depletes human CRP in vivo. Any function proposed for human CRP must be consistent with the remarkable speed and dynamic range of its plasma concentration, which can rise by over 1000-fold in 24–48 h after a strong acute stimulus, such as sepsis or AMI, and can fall with a half time of about 24 h when the stimulus is removed. These dramatic changes are not associated with any local or systemic vascular or inflammatory effects in patients other than those related to the pathology or treatment, which respectively triggered or alleviated the acute phase response.

In patients with established coronary disease, CRP has been shown to predict adverse clinical events. In addition, prospective studies have consistently shown that CRP is a strong predictor of future coronary events in apparently healthy men and women. The relative risk associated with CRP is independent of other CVS risk factors.

Gad et al. reported a specific elevation pattern of CRP that copes with the severity of CAD (Gad et al. 2010). In the same study, the authors noticed the explicit difference in the levels of biomarkers between chronic and AMI CAD patients. Acute patients showed higher serum levels of ADMA, SDMA, and hsCRP and lower serum levels of L-arginine and L-arginine/ADMA ratio. The positive association of SDMA with ADMA in AMI was previously noticed by Korandji et al. (2007), who addressed the suggestion that SDMA could be a good risk indicator for CAD in AMI patients.

The Counter Effect (Vasoconstriction) Arm

Genetic Variation in the Endothelin-1 Gene

Following the discovery of endothelium-derived relaxing factor (EDRF) by Furchgott in 1980 (Furchgott and Vanhoutte 1989), Hickey et al. (1985) published a report that described an endothelium-derived contractile factor. Later, in 1988, this factor was successfully purified, identified as a novel peptide, and named endothelin (ET) (Yanagisawa et al. 1988).

ET-1, which was initially isolated and identified from conditioned medium of cultured porcine endothelial cells, is a potent vasoconstrictive peptide comprising 21 amino acid residues. This peptide has a molecular weight of 2492, free amino and carboxyl termini, and two intramolecular disulfide bonds. ET-1 is present in many mammalian species, including humans. Two additional human endothelin

isopeptides, endothelin-2 and endothelin-3, encoded by separate genes were also detected (Inoue et al. 1989).

As ET-1 plasma concentration is very low; it is not a circulating hormone, but it may be a paracrine/autocrine mediator. ET-1 is released from vascular endothelium and acts on the underlying smooth muscles to increase peripheral vascular resistance. In isolated cardiac muscle, ET-1 induces contraction and exerts a potent positive inotropic action. ET-1 is also reported to induce positive chronotropic action via ETB receptors and negative chronotropic action via ETA receptors. It also controls vascular tone and contraction of myocytes (Miyauchi and Masaki 1999).

However, the precise pathophysiologic effects of ET-1 in AMI patients remain uncertain. In 1994, Omland et al. (1994) reported that the plasma ET-1 level is a prognostic indicator of 1-year mortality after AMI. Animal model also demonstrated that ET-1 may contribute to microvasculature dysfunction due to its potent vasoconstrictive property, thus having adverse effects to AMI by restricting myocardial blood flow following reperfusion (Kelly et al. 1996).

An SNP in exon 5 of endothelin gene (EDN-1) and a G-to-T transversion that causes the Lys-to-Asn substitution at codon 198 have been reported (Tiret et al. 1999). Several association studies tried to explore the relationship between EDN lys198Asn (K198N) polymorphism and cardiovascular diseases, some studies reported an association between this polymorphism and the incidence of hypertension and coronary artery diseases in hypertensives (Tiret et al. 1999; Popov et al. 2008), but others reported lack of association between endothelin gene variants and cardiovascular diseases (Palacin et al. 2009).

Serum Endothelin-1 Levels

Elevated levels of serum ET-1 in MI patients were previously reported (Stewart et al. 1991; Miyauchi and Masaki 1999). ET-1 is increased in accordance with cardiac and pulmonary circulatory distress in AMI patients, which may further aggravate circulatory dysfunction. Stewart et al. demonstrated the early elevation of ET-1 levels after the onset of MI even before the elevation of creatine kinase (Stewart et al. 1991). Several studies showed that the ET-1 level is an important prognostic marker following MI as high ET-1 levels were associated with a higher mortality rate (Omland et al. 1994; Yip et al. 2005). Plasma NO/ET-1 ratio also proved to be efficient for prediction of CAD (Kurita et al. 2005) as the cardiovascular functions are regulated by the balance between the vasodilator NO and the vasoconstrictor ET-1. High ET-1 levels impair endothelial NO production via an isoform-specific PKC-mediated inhibition of eNOS expression (Ramzy et al. 2006).

The origin of the elevated plasma ET-1 post-AMI remains unclear. There is a reason to believe that at least part of this increase originates from the heart, as, in vitro, the ischemic heart releases ET-1 (Brunner et al. 1992), and in the rat occlusion–reperfusion model, plasma ET-1 increases after 50 min of coronary occlusion (Watanabe et al. 1991). ET-1 can be secreted from the atherosclerotic plaque as atherosclerosis precedes AMI. ET-1 is released from activated macrophages and smooth muscle cells, which are abundant in atherosclerotic arteries (Zeiher et al. 1995).

Finally, here are some flashing conclusions of the above reports:

1. *The association of eNOS Glu298Asp polymorphism with CVD is variable among different populations*.
2. *No enough evidence for the correlation between the genotypes of eNOS Glu298Asp and mean serum NO levels*.
3. *Mean serum NO concentrations are different among different populations*.
4. *No age- or sex-related differences in mean serum NO concentrations were observed in healthy subjects*.
5. *A allele/AA genotype for DDAH2 SNP1* (*−1151 C/A*, *rs805304*) *and G allele/GG genotype for SNP2* (*−449 C/G*, *rs805305*) *are associated with early incidence of CAD in Egyptian patients*.
6. *DDAH2 SNP1 and SNP2 are in complete linkage disequilibrium. Association between C/C and A/G alleles for SNP1/SNP2 and CC/CC*, *CA/CG*, *and AA/GG for the genotypes was evident*.
7. *Frequency of SNP1 A allele/AA genotype and SNP2 G allele/GG genotype is directly proportional with the severity of coronary insufficiency.*
8. *No direct association between DDAH2 genotype and serum levels of ADMA*, *SDMA*, *L-arginine*, *and hs-CRP.*
9. *There is a tendency that serum levels of ADMA*, *SDMA*, *L-arginine*, *and hsCRP are correlated with the severity and incidence of CAD.*
10. *C242T polymorphism of the p22 phox gene of NAD(P)H oxidase may reduce susceptibility to MI and that T allele exerts protective effect from CAD*.
11. *Patients having MI had elevated mean serum levels of NO and ox-LDL suggesting the role of inflammation and oxidative stress*, *respectively*, *in the incidence of MI*.
12. *There is a significant correlation between serum levels of NO and ox-LDL in the MI patients*.
13. *Carrying PON1 192R allele represents an independent risk factor for early onset AMI. The PON1 R192 isoform is associated with a higher PON/ARE ratio*.
14. *The PON1 Q192 polymorphism appears to modify the PON-1 enzyme activity.*
15. *Serum hsCRP levels correlate with the severity and incidence of CAD*.
16. *Acute MI patients showed higher serum levels of ADMA*, *SDMA*, *and hsCRP and lower serum levels of L-arginine and L-arginine/ADMA ratio than chronic MI patients*.
17. *There is no enough evidence to prove that EDN Lys198Asn polymorphism is a risk factor for early onset AMI*.
18. *Serum endothelin concentration is higher in MI patients than control subjects*.

Potential Applications to Prognosis, Other Diseases, or Conditions

Until the beginning of the 1980s, nitric oxide (NO) has been just a toxic molecule of a lengthy list of environmental pollutants such as cigarette smoke and smog. In fact, NO had a very bad reputation of being destroyer of ozone, suspected carcinogen, and precursor of acid rain.

However, over the last three decades, the picture has been totally changed. Diverse lines of evidence have converged to show that this sometime poison is a fundamental player in the everyday business of the human body. NO activity was probed in the brain, arteries, immune system, liver, pancreas, uterus, peripheral nerves, lungs, and almost every system in the human body. It exhibits diverse vital roles in the human body. It is now clearly recognized that NO participated in the control of vascular tone as an antagonist of the adrenergic regulatory system. It inhibits aggregation of platelets and their adhesion on a vascular wall. NO causes smooth muscle relaxation not only at the vascular wall but also on the gastrointestinal tract wall. NO functions both in the central and peripheral nervous systems. In brain, it acts as a neurotransmitter and may be a long-sought mystery molecule that aids in learning and remembering. In males, it is the messenger that translates sexual excitement into an erect penis. NO regulates the activity of respiratory system organs and also of the digestive tract and genitourinary systems via efferent nerves. It also influences the functioning of secretory tissues and cells and play roles in vision, feeding behavior, and olfaction. In addition, NO is produced in large quantities during host defense and immunologic reactions.

On the other hand, overproduction of NO is incriminated in the pathogenesis of several conditions such as septic shock, epilepsy, tissue damage, inflammation, and nerve damage left by the stroke. This "split" personality for NO imparted great excitement for more research in this area.

Research on NO has already had a measurable impact on the practice of medicine and drug design, and the impact will increase after understanding all the mechanisms related to NOS activity. Therapeutic strategies related to NO involve both increasing and decreasing NO production. Reduced generation of NO has been implicated in a number of clinical conditions. In these, or even in some situations in which NO production is unimpaired, it may be desirable to mimic or enhance the physiological generation of NO. This may be achieved in several ways, including direct administration of NO, the use of compounds that will donate NO, stimulating receptors linked to the L-arginine–NO pathway, augmenting the action of endogenous NO, or providing additional substrate for its synthesis. On the other hand, inhibition of the synthesis of NO may be desirable in situations in which there is overproduction of NO, as a result of overactivity of any of the NOS isoforms. The analogs of L-arginine are the most commonly used inhibitors of NOS for determining the involvement of NO in a physiological or pathophysiological process. By virtue of their structure similarity to L-arginine, they bind at the substrate-binding site of NOS.

Hundreds of NOS inhibitors are now under development and consideration for their therapeutic potential, especially for inhibition of iNOS that is thought to contribute to the pathophysiology of a number of human diseases, such as arthritis, asthma, inflammatory bowel disease, glaucoma, and psoriasis. However, the world is waiting for a breakthrough in the area of selective NOS inhibition. NOS inhibitors known to date do not possess the desired pharmacological selectivity, pharmacokinetic, and pharmacodynamic profiles to render them therapeutic agents. Clearly, these selective agents will be needed not only as potential therapeutics but also as probes to allow new directions to emerge from the NO research field. Therapeutic

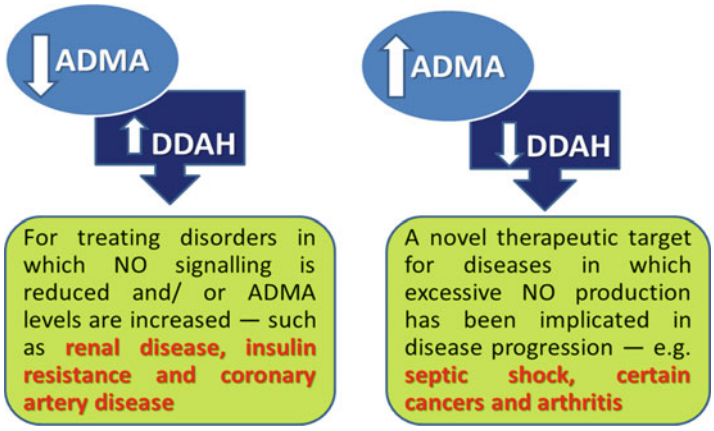

Fig. 8 Therapeutic strategies related to DDAH/ADMA modulation. Asymmetric dimethylarginine (ADMA) is a physiological inhibitor to all nitric oxide synthase isoforms and is degraded by dimethylarginine dimethylaminohydrolase (DDAH). Thus, therapeutic research is going in two directions either by increasing NO as a therapy, through reducing ADMA levels, or by decreasing NO in other conditions by decreasing ADMA levels

strategies related to DDAH/ADMA modulation are also under extensive research (Fig. 8).

Worth mentioning, new directions in this area include trials for gene therapy related to NO generation or inhibition, which have been initiated since two decades and still underway. An example of these trials was done by Kibbe and Tzeng for examining the role of iNOS gene transfer to the vasculature in preventing the development of vascular injury response (Kibbe and Tzeng 2000). Moreover, the importance of NO and ox-LDL is extended from just being targets for prevention, diagnosis, and therapy of CVD to also being candidate biomarkers in evaluating the human biological age (Gradinaru et al. 2015). Association studies of polymorphisms in genes coding for enzymes and proteins metabolizing NO with variable diseases, other than the CVS, are also extensively running (Li et al. 2015; Martinez-Barquero et al. 2015; Polat et al. 2015).

At last, there are still numerous unanswered questions and areas of less understanding that need more research in the next decade, such as:

1. What is the interrelationship between molecular oxygen, NO, and superoxide in normal homeostatic states and in disease?
2. What role does iNOS play in numerous disease pathologies?
3. What is the physiological role of ADMA?
4. What are the reactive nitrogen species directly produced from L-arginine by NOS (NO, nitroxyl, peroxynitrite, or all three) and the stoichiometry of the reaction?
5. What is the significance and the basis of the subcellular localization of the NOSs?

6. What is the molecular and structural basis of the high isoform selectivity of some NOS inhibitors?
7. Will selective iNOS, nNOS, or iNOS + nNOS inhibitors prove to be of value in the treatment of human diseases, and if so, which diseases and what side effects might result?
8. What other physiological and pathological roles of NO in the human body that have not been discovered yet?
9. What are the factors that regulate the metabolism of arginine, the precursor of NOS, and its distribution to different pathways?
10. Which biochemical and genetic biomarkers will be of value in the assessment of cardiovascular health and which of them can be used as early markers of CVD?

Summary Points

- In the blood vessels, nitric oxide (NO) is produced from the endothelium mainly by the constitutively expressed endothelial nitric oxide synthase (eNOS).
- Endothelial NO possesses multiple anti-atherosclerotic properties.
- A range of biochemical disturbances, including reduced availability of NO and oxidative stress, has been shown to be associated with endothelial dysfunction.
- There are several key players that influence vascular NO levels and their role in the protection and/or predisposition to cardiovascular disease (CVD), including the cellular activities of eNOS, dimethylarginine dimethylaminohydrolase (DDAH), paraoxonase, and NAD(P)H oxidase as well as the production levels of asymmetric dimethylarginine (ADMA), oxidized low-density lipoprotein (ox-LDL), C-reactive protein (CRP), and endothelin.
- Alleles or genotypes associated with early incidence of coronary artery disease (CAD) included A allele/AA genotype for DDAH2 SNP1 (−1151 C/A, rs805304), G allele/GG genotype for SNP2 (−449 C/G, rs805305), and carrying PON1 192R allele, whereas C242T polymorphism of the p22 phox gene of NAD(P)H oxidase confers protection from CAD. In contrast, there is no enough evidence to support the association of eNOS Glu298Asp and EDN Lys198Asn polymorphisms on CAD incidence.
- There is a tendency that serum ADMA, symmetric dimethylarginine (SDMA), L-arginine, and hsCRP levels correlate with the severity and incidence of CAD.
- Serum ox-LDL, hs-CRP, NO, and endothelin levels are elevated in CAD patients.

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35 Nonsynonymous Single-Nucleotide Variations as Cardiovascular System Disease Biomarkers and Their Roles in Bridging Genomic and Proteomic Technologies

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_40

Abstract

Non-synonymous single-nucleotide variations (nsSNVs) are mutations in the coding regions of the genome which ultimately lead to amino acid alterations. nsSNVs represent potential diagnostic or therapeutic targets when associated with susceptibility to specific diseases or conditions. The emergence of next-generation sequencing (NGS) technologies has streamlined the process of identifying nsSNVs and offers an avenue for the robust study of disease genetics. This chapter examines the existing roles of nsSNVs in cardiovascular diseases and highlights their values as biomarkers given the current state of research. NGS technologies hold promise for a future of medicine built on understanding the genome and proteome, and the associations of each with disease susceptibility and progression. The chapter also provides an overview of NGS technologies currently available, as well as a sample workflow for harnessing the bio-informational value of nsSNVs.

Keywords

Non-synonymous single-nucleotide variations • Next-generation sequencing technology • Cardiovascular diseases • Biomarker identification • Amino acid variation • Proteomics applications

Abbreviations

CAD	Coronary artery disease
CHD	Congenital heart defects
DCM	Dilated cardiomyopathy
KDR	Kinase insert domain-containing receptor
LOF	Loss-of-function
LQTS	Long QT syndrome
LVEF	Left ventricular ejection fraction
MI	Myocardial infarction
MS	Mass spectrometry
NGS	Next-generation sequencing
nsSNV	Non-synonymous single-nucleotide variations
ORF	Open reading frame
rsIDs	dbSNP database identifier
SCD	Sudden cardiac death
SIDS	Sudden infant death syndrome
SNP(s)	Single-nucleotide polymorphism(s)

SNV	Single-nucleotide variation
VCF	Variant call format
VEGF	Vascular endothelial growth factor
VGSCs	Voltage-gated sodium channel
WES	Whole-exome sequencing
WGS	Whole-genome sequencing

Key Facts of Non-synonymous Single-Nucleotide Variation in Cardiovascular Diseases

- DNA is composed of two strands of repeating nucleotide bases (adenine, guanine, cytosine, and thymine) that make up a "sequence."
- Certain coding regions of DNA encode instructions for proteins such that three adjacent nucleotide bases comprise a codon and determine one amino acid, the building block of proteins.
- Non-synonymous single-nucleotide variations (nsSNV) are changes of a single base in the DNA sequence that result in a different amino acid being produced, and therefore a different, sometimes dysfunctional, protein being produced.
- nsSNVs are known to be associated with human disease, including a number of cardiac diseases.
- Cardiovascular diseases are a set of conditions which affect the structure or function of the heart.
- In addition to lifestyle, obesity, diet, and smoking, genetics are an important risk factor in the development of cardiovascular diseases and conditions.
- Genomics is the study of the entire human genome, or the collection of all genes belonging to a single human.
- Genome sequencing is the process by which the specific composition and order of nucleotide bases in an individual's DNA can be determined.
- A reference genome is an example of a standard genome that is used for comparison purposes – observation of positional differences between an experimentally obtained sequence from an individual and the reference is how nsSNV is discovered.
- Proteomics is the study of the entire human proteome, or the collection of all proteins produced by a single human.

Key Facts of Genomic Variant Discovery

- Next-generation sequencing (NGS) methods are used to discover novel nsSNVs.
- There are many different NGS platforms and new and improved methods are continually being developed.
- The cost of NGS has dropped rapidly from over three billion dollars per human genome to around one thousand dollars currently.

- FASTA and FASTQ file formats are the standards used for recording genome and sequence read information.
- Researchers align short reads to a reference genome in order to generate the genomic sequence of their subject from short reads.
- Coverage depth for a position is the number of short reads resulting from an NGS experiment that cover this position.
- Contigs are continuous regions of the subject's genome that are able to be assembled in the alignment process due to parts of short reads overlapping each other.
- SAM and BAM file formats are the standards used for recording alignment information.
- Single-nucleotide polymorphism (SNP) or single-nucleotide variant (SNV) calling is the process of comparing a given genome (or DNA segment) with a reference genome to determine nucleotide differences.
- The variant call format (VCF) file format is the standard used most often for recording variations (SNVs and larger variations).

Definitions

BAM files Compressed Sequence Alignment/Map (SAM) files.

Biomarker A biological characteristic associated with disease.

Cardiovascular disease Any disease affecting the structure or function of the heart.

Codon Unit of three nucleotides which encodes a specific amino acid based on the nucleotide composition.

FASTA or FASTQ file Next-generation sequencing (NGS) output data of read names and nucleotides, the Q indicates the presence or absence of quality information for each nucleotide read.

nsSNV Variations in coding regions of a genome which result in amino acid substitution.

SAM files Sequence Alignment/Map; Human readable output files of all the read sequences, where they map to the reference genome, and their mapping score.

Introduction

Non-synonymous single-nucleotide variations (nsSNV) are mutations in the exonic or coding regions of the genome which, when transcribed and then translated, lead to substituted amino acids (missense mutations) or truncated proteins

(nonsense mutations). These alterations in the amino acid sequence may influence protein folding, disrupt protein-protein interactions, or even directly modify the active site (Dingerdissen et al. 2013). nsSNVs are not the only type of genetic mutation, but they are particularly valuable biomarkers and, due to their potential effects on protein function, represent a starting point for investigating biochemical pathways. Although this chapter focuses primarily on nsSNVs of the missense type, it is important to note that both missense and nonsense variations cause changes in the protein sequence, with respect to the normally translated protein, and should therefore be detectable by the proteomic technologies discussed below.

Next-generation sequencing (NGS) methods are essential in the search for nsSNVs as biomarkers for all aspects of physiology, including the cardiovascular system. There are several platforms that generate NGS data, and there is the promise of new, so-called ultrarapid technologies like nanopore sequencing (Deamer and Akeson 2000) on the horizon. Major software developments have addressed the complex computational challenges which stemmed from the extra-large scale of genomic data generated by NGS technologies. These tools facilitate the assembly and alignment of NGS data, the subsequent calling of single-nucleotide polymorphisms (SNPs), or the identification of other types of genomic variation in a sample. Determination of biomarkers from the pool of variation requires the integration of additional software developments with statistical analysis and a detailed consideration of disease-related annotations.

While genomic strategies have provided a broad foundation for the cataloging of disease-associated nucleotide variation, newly developed high-throughput proteomic technologies (Branca et al. 2014) can further elucidate biological and physiological understanding of amino acid variation at a molecular resolution (National Research Council 2006). Quantitative and structural proteomic approaches have already been applied to variant-based biomarker discovery in a number of human diseases (Nie et al. 2014; Marrocco et al. 2010) and hold the same promise for cardiovascular biomarker identification.

Diseases of the cardiovascular system affect the structure and/or function of the heart: they include conditions such as heart failure, sudden cardiac death (SCD), and coronary artery disease (CAD). Altogether, cardiovascular diseases are the leading cause of death for both men and women in the United States (Mozaffarian et al. 2015). While the causes and risk factors behind specific conditions are varied and multifaceted, it is agreed that genetics plays an influential role in susceptibility. Consequently, the ability to identify nsSNVs quickly and accurately is valuable toward the further study of the origins and outcomes of these often fatal conditions. As NGS technologies continue to improve, nsSNVs may play an increasingly important role as therapeutic and diagnostic biomarkers in cardiovascular system diseases. This chapter will offer a brief introduction to the roles of nsSNVs across conditions and diseases under the umbrella term of cardiovascular systems diseases.

Technologies Used in Variant Detection

The first full human genome was sequenced by a chain-terminated (Sanger) sequencing method (Sanger et al. 1977) and cost approximately three billion dollars. The high cost and time-intensive nature of sequencing prevented widespread use of the technique until the discovery and development of new massively parallel sequencing methods (Metzker 2010; Grada and Weinbrecht 2013), later termed next-generation sequencing methods. Massively parallel throughput systems take advantage of the speed and efficiency of sequencing genetic fragments in parallel and then reassemble them via computational alignment algorithms. Although initially very expensive, the costs of these sequencing methods have fallen drastically, approaching $1,000 per sample, bringing the goal of personalized genomic medicine closer than ever before.

NGS methods generally produce large series of short reads, often between 75 and 300 bases in length, depending on the machine used (Metzker 2010). It is not uncommon to produce over one billion short reads in a single experimental run. Although this massive volume of data presents computational challenges, finding efficient solutions is essential as the number of entities, both research and clinical, generating and using NGS data is rapidly expanding (Metzker 2010).

Several major platforms are currently available for next-generation sequencing including Pacific Biosciences, Ion Torrent, Roche/454, Illumina/Solexa, and SOLid, while exciting new techniques such as nanopore technology are undergoing development. This improved technology shows promise in producing very long read lengths (~10 kb or higher) to address current limitations to *de novo* assembly and alignment of sequence reads to a reference genome (Wang et al. 2014).

The basic pipeline of variant detection is shown in Fig. 1. The pipeline becomes increasingly complicated when augmented with additional quality control and analysis steps, but the schematic presented herein represents the core of the variant calling process.

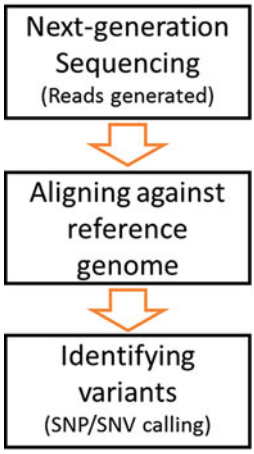

Fig. 1 Basic variant calling pipeline starting from NGS

Mapping of Reads (Generating an Alignment)

A NGS experiment usually produces a FASTQ file which can then be mapped to a reference genome in FASTA format. A FASTA file contains only the read names and the nucleotide sequence of that read with a single file containing records for up to millions of reads. A FASTQ file contains the same ID and read information plus quality information for each nucleotide position as determined by the machine used. This quality information represents the confidence that a particular nucleotide was correctly identified by the sequencing machine.

Since short genomic reads are produced with variable coverage depth at any given position, reads are mapped, or aligned, to a reference genome. Generally, the human reference genome published by the Genome Reference Consortium (http://www.ncbi.nlm.nih.gov/projects/genome/assembly/grc/) is used for human samples. After specifying the reference genome, software maps each read to the genome via a computational alignment algorithm that takes a read and determines the most likely coordinates from the genome from which the experimental read was obtained. Coverage is determined for each nucleotide location that has been sequenced and can be matched to a read. Various software packages have been developed for this task, including BLAST (Schuler et al. 1991), TopHat (Trapnell et al. 2009), BWA (Li and Durbin 2009), HIVE-hexagon (Santana-Quintero et al. 2014), and others.

After alignment is complete, the total number of reads that were successfully aligned to a given position make up the coverage depth for that position, with full coverage of the experiment represented by the average coverage across all positions (see Fig. 2). Ideally, the genome will be fully covered such that overlapping reads map from one end of the genome (or chromosome) to the other without any gaps. However, this is frequently not the case, so the alignment software will also report the number of contigs – continuous regions of coverage provided by the reads. Some positions will have greater depth than others as an artifact of sequencing chemistry. This is an important consideration for assessing nsSNV as coverage is inferred to provide direct evidence for the presence of a variant in a sample.

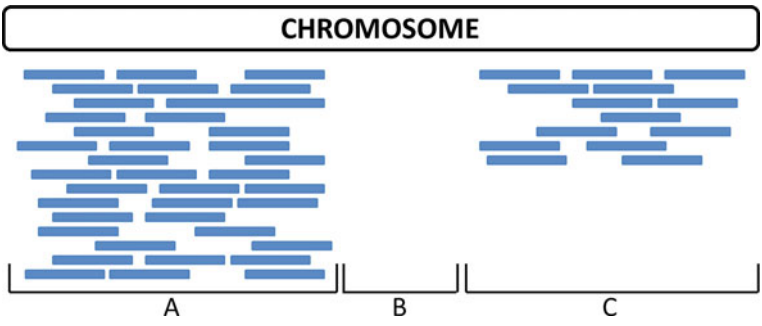

Fig. 2 Read mapping to a reference genome. (**a**) High coverage area of the chromosome; (**b**) Area of chromosome with no coverage; (**c**) Area of genome with low coverage

The output formats for this process vary widely, but the most common formats are SAM/BAM alignment files. Sequence Alignment/Map (SAM) files are human readable, whereas BAM files are compressed versions of the same information. Both of these files contain all the read sequences as well as where they map to the reference genome and their mapping scores (a measure of how well they mapped).

SNV/SNP Calling

Software is then used to "call" the variants at reported positions. Variant positions are those where the mapped nucleotides differ from the expected reference nucleotide. Common software used for variant calling includes SAMtools (Li et al. 2009a), HIVE-heptagon (Simonyan and Mazumder 2014), and SOAP2 (Li et al. 2009b). The variant calling process is more complicated in diploid and other polyploid organisms which can have two different nucleotides at a single position due to multiple copies of chromosomes, or by sequencing errors inherent in the process. A clever algorithm can utilize a higher coverage level to discard erroneous variations and also report the proportions of nucleotides in specific positions. For a human heterozygous at the position of interest, one would anticipate two different nucleotides each appearing 50 % of the time throughout the coverage. For a human homozygous at the position of interest, one would expect a single nucleotide to be represented. It is, however, possible to have a mosaic set of DNA from an individual or for nucleotides to be inserted or deleted relative to the reference genome.

After variant calling, a file is produced cataloging the variations found in the alignment. The most common format is the Variant Call Format (VCF) file. This is a human readable file which contains information about the position of each call as well as the reference nucleotide(s), the variation(s) noted including insertions and deletions (commonly called indels), and the frequencies of each variation. Additional optional, user-defined information can be included depending on the specifications of the researcher.

Identifying nsSNVs

Once variants are called, it is possible to categorize each single-nucleotide variation (SNV) as either non-synonymous or synonymous. Software is used to look at each variant's position and compare that to a database of coding regions. The database contains information regarding open reading frames (ORFs) of the coding region which host the SNV. With information about the open reading frame, the software is then able to determine the new codon when the reference nucleotide is replaced by the variant one. This three-letter nucleotide set codes for the amino acid that will be included in the protein. Depending on the location of the nucleotide change, the amino acid might also change (e.g., often if the variation is in the first position of the codon) or it might remain the same (most commonly when the change occurs in the final position of the codon).

If the amino acid changed due to the variation, then the SNV is called a non-synonymous variation (see Fig. 3). Non-synonymous variations have the

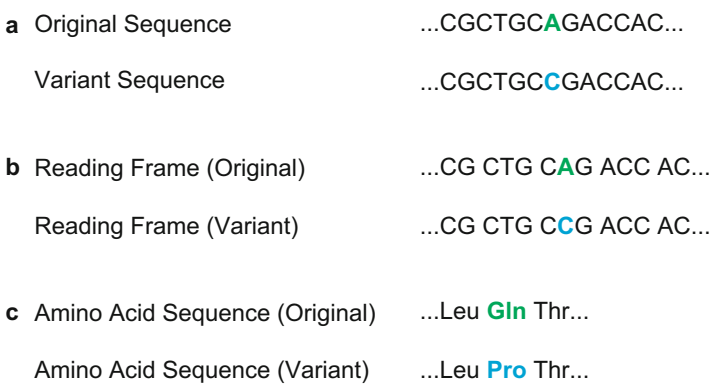

Fig. 3 (**a**) The original DNA sequence of the sense (coding) strand in the 5' to 3' direction followed by the sequence with the SNV (the variant sequence) in the same orientation; (**b**) The sequences from A with the ORF information added; each space separates the nucleotide codons, or set of three nucleotides that code for an amino acid; (**c**) The amino acid sequence translated from mRNA transcribed from the original and variant sequences; here, the original sequence results in the amino acid chain leucine – glutamine – threonine. The variant sequence results in the amino acid sequence leucine – proline – threonine. In this example, the SNV would be considered non-synonymous since leucine was changed to a completely different amino acid, proline, due to the variation

potential to affect the function of the protein by direct interruption of active or binding sites or by indirect effects such as steric hindrances, charge modifications, and others. Disruption of the protein can also happen when the new amino acid changes the protein's three-dimensional structure. Synonymous variations, on the other hand, are generally innocuous. They do not directly change the shape or function of the protein, but can have regulatory effects by changing the rate that RNA polymerase is able to transcribe the region or by altering binding properties of that portion of the DNA.

From Genomic to Proteomic Identification

Since the advent of NGS technology, identification of genomic variation through whole-genome sequencing (WGS) and whole-exome sequencing (WES) has greatly improved, enhancing the ability to study genotype-phenotype disease associations. The International HapMap Project (International HapMap et al. 2007) has contributed to the identification of approximately ten million common DNA variants, primarily SNVs. Despite this accomplishment, however, the project asserts that current knowledge of human genetic variation is incomplete due to lack of information about rarer variants, such as minor allele frequency variants and copy number variants, which are not as well studied (International HapMap et al. 2010). Pilot results of the 1000 Genomes Project (Genomes Project et al. 2012) also demonstrate the limitations of genomic approaches, indicating that, while much common variation has been captured, significant phenotypic variation can be attributed to variants missed by commonly used genotyping arrays.

Thus, while genomic strategies have laid the foundation for the cataloging of variation, disease-associated and otherwise, there is still much left to be discovered. Furthermore, drawbacks of whole-genome approaches include high costs and provision of an overwhelmingly vast amount of data, increasing the difficulty of discerning benign variants from those that may be pathogenic (Royer-Bertrand and Rivolta 2015). Technical aspects of NGS data also present significant challenges including storage and maintenance, quality control, and analysis that is both reliable and efficient (Xuan et al. 2013). Despite disadvantages of genomic strategies of variant detection, the knowledge that can be deduced from such studies is imperative to a complete understanding of certain disease states.

Similarly, proteomic technologies used to discover disease-associated amino acid variation biomarkers are greatly beneficial. High-throughput proteomic technologies have only recently been developed (Branca et al. 2014), but have the potential to enable an understanding of biological and disease processes with increased granularity as compared to genomic technologies. While these strategies may pose similar challenges to technical logistics as described for the genomic approaches, the knowledge gained from proteomic studies is closer to the pathology of complex disease states and therefore closer to disease detection and therapy (National Research Council 2006). To this end, several proteomic databases have emerged over the last decade including GPMDB (Craig et al. 2004), PeptideAtlas (Deutsch 2010), MassIVE, Chorus, PRIDE (Cote et al. 2012), and more. Many of these databases belong to the ProteomeXchange consortium (www.proteomexchange.org) which facilitates central access to shared data across resources and maintains guidelines for acceptable data formatting. Despite best efforts, a number of unique challenges exist such as MS/MS spectral and peptide database matching, incomplete sequence databases with missing or incorrect annotations, the need for optimization, and the lack of standardized preparation and validation protocols (Omenn et al. 2005). As databases evolve to include a more biologically representative set of viable proteins and synthetic constructs of potential variant including peptides, the quality of resultant peptide libraries will increase tremendously. In turn, it will become easier to analyze the presence and statistical importance of nsSNVs as potential disease biomarkers.

Some current applications of proteomics to nsSNV-based biomarker discovery include quantitative analysis of pancreatic cancer-associated single-amino-acid variant peptides (Nie et al. 2014), identification of cancer-related splice variants and validation via custom library (Hatakeyama et al. 2011), and discovery of a novel hepatitis B-related candidate biomarker (Marrocco et al. 2010). There is a great emphasis in the literature and interest in the community on best interpretation of quantitative analysis, methods for identifying low-abundance peptides, and custom-built, purpose-specific peptide databases. With respect to cardiology, a combined tandem mass spectrometry and sequence homology approach was used to identify a novel, single-amino-acid variation resulting from nsSNV in swine cardiac troponin I (Zhang et al. 2010). These cases demonstrate the enormous potential of proteomics to further resolve mechanisms of various cardiovascular diseases and identify single-amino-acid variation resulting from nsSNVs as diagnostic biomarkers or potential therapeutic targets.

Potential Applications to Prognosis, Other Diseases, or Conditions: Cardiovascular Diseases and Associated nsSNVs

The following sections explore the different cardiovascular diseases and associated nsSNVs. While each of these conditions can be characterized by a wide range of biomarkers, symptoms, and risk factors, the nsSNVs reported were found to be associated with the disease, either through increased susceptibility or even decreased susceptibility. The nsSNVs are potential points for further investigation and do not yet represent definite clinical diagnostic markers. In the following text, specific variants are referred to by the rsID, or the reference SNP cluster ID, which is the accession number for a given variant in the dbSNP database.

Ischemic Stroke

An ischemic stroke is a lack of blood reaching the brain and is caused by narrowing or clogging of blood vessels with plaque (American Stroke Association 2013). According to stroke.org, someone dies from stroke every 4 min in the United States, and stroke is also the leading cause of adult disability. Ischemic stroke is associated with high mortality and severe morbidity: victims often experience permanent neurological disability following an episode (Lee et al. 2010). The main risk factors of ischemic stroke are high blood pressure, high cholesterol, and diabetes, but research suggests that genetic variations are another important factor (Flossmann et al. 2004; Gretarsdottir et al. 2003). While the exact mechanisms by which genetic variations influence the likelihood of ischemic stroke are poorly understood, the associations are significant (Guo et al. 2013).

In a recent study of 1,209 patients with stroke and 1,174 controls from a Chinese population, researchers found that rs2230500 is significantly associated with both the risk of ischemic stroke (age- and sex-adjusted odds ratio = 1.37; 95 % CI, 1.12–1.67; $P = 0.0019$) and cerebral hemorrhage (age- and sex-adjusted odds ratio = 1.96; 95 % CI, 1.21–3.19; $P = 0.0064$) (Wu et al. 2009). This result confirmed previous studies finding the variant significantly associated with stroke in Japanese populations (Kubo et al. 2007; Serizawa et al. 2008). Note that both of these are Asian populations where the minor allele frequencies of this nsSNV are 0.239 for Japanese in Tokyo and 0.178 for Han Chinese in Beijing. According to the HapMap database, the minor allele frequencies for Utah residents with Northern and Western European origins was 0.008, and 0.00 for Yoruba in Ibadan, Nigeria (Kubo et al. 2007). The polymorphism is a G to A substitution in exon 9 at position 1425 of PRKCH, a gene located in position 61457521 of chromosome 14q22–q23 in humans. The variant causes an amino acid substitution from valine to isoleucine in position 374 of the protein (Shimizu et al. 2007).

The residue change occurs in the ATP-binding site of the serine-threonine kinase (Wu et al. 2009). PRKCH is known to be involved in a variety of signaling pathways

and regulates cellular functions such as proliferation and apoptosis (Kubo et al. 2007). Expressed mainly in endothelial cells, the kinase plays a role in human atherosclerosis (Kubo et al. 2007). The nsSNV was found to significantly increase autophosphorylation and kinase activity after stimuli (Kubo et al. 2007). This agrees with the biological plausibility of the assertion that if a protein involved in atherosclerosis, a risk factor of ischemic stroke, is overly activated due to a genetic mutation, there will consequently be a higher risk of stroke.

Coronary Artery Disease

Coronary artery disease (CAD) is the most common type of heart disease and is responsible for the most deaths in the United States among men and women every year (National Heart Lung and Blood Institute 2014). The disease is characterized by the accumulation of plaque in the coronary arteries (National Heart Lung and Blood Institute 2014). This process, called atherosclerosis, gradually deprives the heart of oxygen-rich blood over time. If incoming blood is sufficiently blocked, a heart attack will occur. The major risk factors of coronary artery disease include dyslipidemia, smoking, hypertension, and diabetes (Achari and Thakur 2004). Unfortunately, due to the complexity of CAD, the influence of genetic factors on disease susceptibility is not completely understood. Pathogenesis is believed to be caused by the interactions of multiple genetic and environmental influences. The major role family history plays as an indicator of CAD susceptibility strengthens the idea that a genetic component is important (Wang 2005).

One potential genetic biomarker is the nsSNV rs2305948 on chromosome 4 at position 55113391 (Sherry 2001). The role of the polymorphism as a risk indicator for CAD was confirmed in two independent case–control studies. The first study was comprised of 655 patients with coronary heart disease and 1,015 controls, whereas the second study was based on 369 subjects and 625 controls (Wang et al. 2007). The two studies found that rs2305948 is associated with risk of coronary heart disease with an odds ratio of 1.41 ($P = 0.011$) in the first cohort and an odds ratio of 1.75 ($P = 0.003$) in the second cohort (Wang et al. 2007). The polymorphism is a C to T substitution in exon 7 of the kinase insert domain-containing receptor/fetal liver kinase-1 (KDR) gene. KDR is a receptor for the vascular endothelial growth factor (VEGF): together, they play a critical role in angiogenesis and vascular repair. The variant in the KDR gene results in an amino acid substitution from valine to isoleucine in position 297 in the third NH_2-terminal Ig-like domain within the extracellular region (Wang et al. 2007). As a key component of the VEGF-binding domain, the nsSNV decreases the efficiency of VEGF and KDR binding. This inhibits KDR function and dampens the resulting signaling pathway (Wang et al. 2007). While recent experiments in animal models have shown that VEGF promotes atherosclerosis, the exact mechanism by which KDR influences disease development is still unknown (Wang et al. 2007).

Sudden Cardiac Death

Sudden cardiac death (SCD) is estimated to be involved in a quarter of all human deaths globally each year (Abhilash and Namboodiri 2014). SCD describes an unexpected death within an hour of symptom onset due to cardiac causes without any extra cardiac event having occurred within the previous 24 h (Havmoller and Chugh 2012). While most instances of SCD are caused by ventricular fibrillation (Abhilash and Namboodiri 2014), other risk factors include coronary heart disease, physical stress, structural changes in the heart, and inherited disorders (National Heart Lung and Blood Institute 2011). Low survival rates have catalyzed the effort to identify improved risk markers (Havmoller and Chugh 2012). While the current widely used risk markers include QT interval and LVEF, the addition of potential biomarkers such as plasma and inflammatory markers has yet to provide adequate predictive value (Havmoller and Chugh 2012). However, the use of genomic or proteomic technologies may supply novel diagnostic and therapeutic targets.

One potential marker is the variant rs7626962 found on chromosome 3 in position 38579416. Although the variant has a minor allele frequency of approximately 13 % in African American populations (Cheng et al. 2011), it is difficult to conduct a genome-wide association study on deceased patients. Consequently, many variations are discovered in postmortem genetic testing. One association was found in a genetic analysis of a 23-year-old African American male who died suddenly (Cheng et al. 2011). The variant was also found in three affected members of a white family but not found in the non-affected family members (Chen et al. 2002). This finding is especially significant as the polymorphism was understood to have negligible prevalence in populations of white European ancestry (Splawski et al. 2002). Furthermore, two separate studies confirmed the association of rs7626962 with sudden death. The first examined 133 cases of sudden infant death syndrome (SIDS) and 1,056 controls and found that infants with two copies of the polymorphism have a 24-fold increased risk for SIDS (Plant et al. 2006). The second study also found a significant association between the nsSNV and SIDS in a cohort of 71 African American SIDS victims (Van Norstrand et al. 2008).

The variant is a C to A mutation in position 3308 of the SCN5A gene and causes an amino acid change from serine to tyrosine in position 1103 of the protein (Cheng et al. 2011). SCN5A is a voltage-gated, type V, alpha subunit sodium channel (Sherry 2001). In addition to sudden cardiac death, mutations in this gene are known to cause Brugada syndrome, long QT syndrome (LQTS), and arrhythmias (Abunimer et al. 2014; Plant et al. 2006). Although experiments showed that mutant and wild-type variants of the sodium channel behave identically at pH 7.4, functional differences were observed when tested under conditions that would be expected *in vivo*. When pH was decreased from 7.4 to 7.0 and then 6.7, as would be expected in acidosis, the Y1103 variants experienced progressive shifts in the voltage dependence of steady-state inactivation. In addition, the mutant channels had shortened recovery times from inactivation. This suggests that, in conditions of low internal pH, mutant SCN5A channels may activate during unanticipated periods of the

cardiac cycle compared to wild-type channels. This hypothesis was confirmed when the variant channels were found to abnormally reopen during depolarization at pH 6.7 compared to wild-type channels which remained inactive (Plant et al. 2006). This unexpected opening of sodium channels, which play a crucial role in cardiac cycles, may explain the association of the nsSNV and SCD, as well as provide further evidence to the value of rs7626962 as a biomarker in assessing SCD preventative therapy.

Congestive Heart Failure

In 2009, one in nine deaths in the United States was partially linked to heart failure. Today, there are approximately 5.1 million people living with heart failure, and nearly half of people who develop heart failure die within 5 years of diagnosis (Go et al. 2013). Risk factors for the disease include coronary heart disease, high blood pressure, diabetes, smoking, poor diet, sedentary lifestyle, and obesity. While it is known that there is a strong hereditary component, this component is poorly defined in common forms of the disease (Cappola et al. 2011). One promising genetic marker is a loss-of-function (LOF) variant in the CLCNKA chloride channel.

The nsSNV rs10927887 was found to be positively associated with heart failure in three independent Caucasian heart failure populations. The variant on chromosome 1 in position 16024780 is an A to G substitution in the CLCNKA gene, which leads to an arginine to glycine change in position 83 (exon 3) of the protein (Sherry 2001). The variant was found to be present in 50 % of the 625 unaffected controls and in 56 % of 1,117 Caucasian heart failure cases. These frequencies were similar in examination of another independent cohort of 857 subjects and 311 controls. The association was robust enough to be statistically significant in a subgroup analysis for heart failures of any type. Independent of age, gender, and hypertension, the risk of heart failure increases by 27 % and 54 % for heterozygotes and homozygotes of the nsSNV, respectively (Cappola et al. 2011). These associations are likely a result of the functional differences in the ClC-K_a channel as a result of the amino acid substitutions. The glycine 83 mutant channels evoked currents with smaller amplitudes across tested potentials compared to wild-type channels. In addition, the efficiency of the mutant channels was less sensitive to extracellular chloride ion concentration compared to wild type. An immunoblot analysis used as a control found no difference between expression levels of the two channels in the cellular model, suggesting that any differences in efficacy was due to the inherent characteristics of the mutant channels (Cappola, Matkovich et al. 2011). Ostensibly, a nsSNV reducing the chloride currents through a renal ClC-K_a chloride channel would not cause congestive heart failure. However, a known variant, Cys 80 ClC-K_a mutation, with a similar LOF profile was found to cause a Bartter-like syndrome in conjunction with the disruption of the related CLCNKB gene (Schlingmann et al. 2004). This syndrome is a salt-wasting disorder of which one abnormality is hyperreninemia, an established risk factor for heart failure (Modlinger et al. 1973; Bongartz et al. 2005).

Myocardial Infarction

Every year in the United States, an estimated 785,000 people will have a new myocardial infarction (MI). With approximately a death every minute in the United States, MI is a major cause of morbidity globally (Jneid et al. 2013) and the leading cause of death among all cardiovascular diseases (Sahoo and Losordo 2014). While the exact definition of a myocardial infarction includes patient symptoms, echocardiogram changes, and sensitive cTN biochemical markers, it is, in essence, a condition in which inadequate blood flow to heart muscles disrupts cardiac function and prompts necrosis (Jneid et al. 2013; National Heart Lung and Blood Institute 2013). Risk factors for MI include controllable risk factors such as smoking, hypertension, high cholesterol, obesity, a sedentary lifestyle, and uncontrollable factors such as age and genetics.

One possible biomarker in assessing the risk for myocardial infarction is the SNP rs73184536. While most of the nsSNVs explored in this chapter increase risk of a cardiovascular disease or condition, this variant offers protection. Found on chromosome 13 in position 37636968, the variant codes for a T to C allelic substitution in the gene for the transient receptor potential cation channel, subfamily C, member 4 (TRPC4). This mutation in exon 11 results in an isoleucine to valine substitution at position 957 of the protein (Jung et al. 2011). In a sample of 3,899 controls and 1,025 patients with a first MI, the variant was associated with decreased risk of MI (odds ratio = 0.61; 95 % CI (0.40–0.95); $P = 0.02$) when adjusted by age, sex, hypertension, and antihypertensive therapy.

The gene belongs to a family of nonselective ion channels and is expressed in vasculature (Yip et al. 2004) where it facilitates intracellular Ca^{2+} signaling. Intracellular Ca^{2+} signals are critical in the regulation of endothelial permeability (Tiruppathi et al. 2002), smooth muscle proliferation (Zhang et al. 2004), and endothelium- and nitric oxide (NO)-dependent vasorelaxation (Freichel et al. 2001). As mentioned before, the crux of the problem in MI is the inhibition of blood supply to the myocardium. As blood is a liquid, flow is inversely related to the resistance from the myocardial vascular bed (Jung et al. 2011). This resistance is dependent on the vascular smooth muscle and consequently on calcium signaling (Jaggar et al. 2000). TRPC4 activity is regulated through kinase phosphorylation of a tyrosine in position 959 that, once activated, inserts additional channels into the plasma membrane (Jung et al. 2011). A single-channel analysis revealed a threefold increase in active TRPC4-I957V channels compared to wild-type channels following carbachol stimulation. The enhanced channel activity of the TRPC4 variant increases Ca^{2+} signaling which may facilitate endothelium- and NO-dependent vasorelaxation. This process may ultimately decrease resistance in the myocardial vascular bed and explain the MI risk protection offered by the nsSNV rs73184536 (Jung et al. 2011).

Congenital Heart Defects

According to the American Heart Association, congenital heart defects are a common form of birth defects and comprise a long list of heart malformations, including

aortic valve stenosis and atrial septal defect. Every year in the United States, nearly 1 % of births are affected by congenital heart defects (CHD). While not all cases are fatal, CHDs are responsible for 4.2 % of all neonatal deaths. In addition, while 95 % of babies born with a noncritical CHD are expected to survive to adulthood, this increases the number of adults living with CHD (Center for Disease Control and Prevention 2014). The exact mechanisms behind each type of defect vary, but CHDs are generally understood to be a result of multiple environmental and genetic factors (Arrington et al. 2012).

One potential genetic marker is a non-synonymous mutation in the pre-B-cell leukemia homeobox 3 (PBX3) gene. The rs145687528 variant is found on chromosome 9 in position 125915818 and is a C to T substitution which results in an alanine to valine amino acid substitution in position 136 of the protein (Sherry 2001). The variant is positioned in a conserved polyalanine track and was present in 5.2 % of the 95 heart defect patients, compared to only 1.3 % of the race and ethnicity-matched control patients (Arrington et al. 2012). This significant overrepresentation of the variant reveals rs145687528 as a valuable risk allele for congenital heart defects (Arrington et al. 2012).

The gene in question codes for a pre-B-cell leukemia homeobox (PBX) protein and belongs to the pre-B-cell leukemia (PBC) transcription factor family and shares a three-amino-acid loop extension in the homeodomain with other members of the TALE superfamily (Arrington et al. 2012). The variant is the seventh alanine in a nine-alanine motif in PBX3. That the amino acid sequence is highly conserved bolsters conclusions from *in silico* analysis which showed a high probability that the mutation is deleterious (Arrington et al. 2012). Polyalanine tracts are thought to be involved in transcription factor repression or facilitation of DNA binding in a transcription complex (Brown and Brown 2004). While the exact mechanism by which this mutation leads to a congenital heart defect is not understood, it does provide a new avenue for further investigation.

Hypertension

Hypertension is a chronic condition where elevated blood pressure slowly damages blood vessels and organs. The increasing rate of hypertension is a cause for concern as it is a major risk factor for cardiovascular disease and leads to higher mortality globally (Lawes et al. 2008; Xi et al. 2012, 2013). Currently, an estimated 26.4 % of the world's adult population are afflicted with hypertension (Kearney et al. 2005). While obesity, stress, and excess salt in the diet are known causes of hypertension, there are also genetic factors that interact and play a role (Medicine 2015). Genetic factors contribute approximately 20–40 % of the variance in blood pressure among the general population (Choh et al. 2005). Another approximation attributed 65 % of variation in blood pressure over a 24 h period to genetic factors (Tobin et al. 2005).

One potential genetic factor is the nsSNV rs7565062 in the gene SCN7A. Found in exon 25 on chromosome 2 in position 166477575, the variant is a G to T substitution that leads to a threonine to asparagine change in position 41 of the

sodium channel, voltage-gated, type VII, alpha subunit (Sherry 2001). In a study of 1,232 unrelated subjects from the Northern Han population of China, 615 with hypertension and 617 controls, the T allele in rs7565062 had significantly higher prevalence in the hypertensive cohort ($P = 0.045$). This association with hypertension signifies that the T allele acts as a risk factor for the condition. Through logistic regression analysis, rs7565062 was found to be significantly associated with essential hypertension in both the additive (TT vs. TG vs. GG: $P = 0.024$, OR = 1.283, 95 % CI: [1.033–1.592]) and dominant ((TT + TG) vs. GG: $P = 0.013$, OR = 1.203, 95 % CI: [1.040–1.392]) genetic models (Zhang et al. 2015).

The sodium channel, voltage-gated, type VII, α-subunit (SCN7A) belongs to the gene family encoding the α-subunit of voltage-gated sodium channels (VGSCs). Although this is the official classification of the channel, one study found the channel encoded in part by SCN7A is sodium concentration gated rather than voltage-gated (Hiyama et al. 2002). The channel was also identified to function as a sodium-level sensor in blood flow (Shimizu et al. 2007) and regulate sodium intake (Hiyama et al. 2010). The mechanism in which this variant induces hypertension may be found through its connection with Na_x, which is an isoform of the α-subunit found in voltage-gated sodium channels (Zhang et al. 2015). Na_V2 is a member of the SCN7A-encoded Na_x and is expressed in the neurons and ependymal cells in circumventricular organs involved in body-fluid homeostasis (Watanabe et al. 2000). Experiments in a mouse model showed that Na_x-null mice had abnormal intake of hypertonic saline. The finding suggests that Na_x monitors sodium concentration and is involved in sodium intake regulation (Zhang et al. 2015). These findings offer a biological context that reinforces the association between the mutation and elevated risk of hypertension.

Arrhythmia

Arrhythmias, including atrial fibrillation, tachycardia, and bradycardia, are a set of conditions defined by abnormal electrical activity of the heart and are a major cause of stroke and sudden cardiac arrest (Abunimer et al. 2014). The role of arrhythmias in these sudden adverse cardiac events is such that hereditary arrhythmias are responsible for over half of sudden cardiac deaths in young individuals (Beckmann et al. 2011). Despite the low prevalence of hereditary arrhythmias in populations, early detection of the condition is essential to beginning early preventative measures. Consequently, understanding the genetic causes underlying the various conditions categorized as arrhythmias is imperative for improving diagnosis and therapy and ultimately identifying individuals who may be at a higher risk for severe cardiac events associated with arrhythmias.

A candidate for further genetic study of arrhythmias is the nsSNV rs6795970. A study found the mutation strongly associated with QRS duration, which measures cardiac intraventricular conduction and is a common indicator of arrhythmias (Ritchie et al. 2013). The exact mutation is an A to G substitution in the sodium channel, voltage-gated type X, alpha subunit (SCN10A) gene, in chromosome 3 at

position 38725184. This exonic polymorphism corresponds to a valine to alanine amino acid substitution at position 1073 (Sherry 2001). In a phenome-wide association study of nearly 14,000 European-American subjects, this particular SNP on chromosome 3 was found to be significantly associated with cardiac arrhythmias, atrial fibrillation and flutter, arterial embolism and thrombosis, and many other conditions. While the association with cardiac arrhythmias was strongest, the association of rs6795970 with altered QRS duration and with cardiac arrhythmia were not dependent, which suggests that while the SNP may influence QRS duration and susceptibility to arrhythmia development, their pathways are divergent (Ritchie et al. 2013).

The gene in question, SCN10A, is a voltage-gated sodium channel labeled $Na_V1.8$ and codes for a protein more commonly known for cold perception in afferent nociceptive fibers (Blasius et al. 2011). While the exact mechanism through which the mutated SCN10A gene leads to arrhythmias is unknown, the three predominant theories are that it affects conduction directly via cardiomyocytes, indirectly via intracardiac neurons, or, more recently proposed, as an enhancer of SCN5A gene expression. A recent study discovered that while SCN10A expression is negligible in human and murine hearts, a T-box enhancer within SCN10A drives SCN5A expression in cardiomyocytes (Park and Fishman 2014). This third theory is further evidenced by previously inconclusive studies of attempting to characterize the role of the SCN10A protein in heart physiology (Akopian et al. 1996). Despite a yet uncharacterized pathway, the nsSNV rs6795970 is definitively associated with cardiac arrhythmias, and further study on the SNP is necessary to further elucidate potential therapeutic or diagnostic targets.

Cardiomyopathy

Cardiomyopathies are diseases of the myocardium classified by structural and functional abnormalities (Sisakian 2014). In most cases, heart muscle becomes thicker or more rigid than normal. While patients with cardiomyopathy may live long healthy lives, it is a major cause of heart failure which is a leading cause of death (Simonson et al. 2010). As with other conditions and diseases, genetic biomarkers are playing an increasingly important role in classification and diagnosis (Sisakian 2014).

One potential marker for identifying susceptibility for dilated cardiomyopathy (DCM) is the cytotoxic T-lymphocyte antigen 4 (CTLA4) (Ruppert et al. 2010). The receptor belongs to the CD28-B7 immunoglobulin superfamily of immune regulatory molecules which downregulate T-cell activation. CTLA4 is expressed on the plasma membrane of activated T cells and functions as an inhibitory signal for T-cell proliferation after binding to B7 receptor molecules on antigen-presenting cells (Ruppert et al. 2010). Ostensibly, a receptor in the immune system should not be involved in the development of DCM. However, a major factor in DCM pathogenesis is known to be autoimmune-mediated damage to cardiac tissue (Ruppert et al. 2010).

The mutation in question is rs231775, an A to G substitution in position 49 of exon 1 of CTLA4 on chromosome 2 in position 203867991 (Sherry 2001). The nsSNV was confirmed in a study of two independent cohorts of dilated cardiomyopathy patients ($n = 251$ and 223) and a sample of 591 healthy controls (Ruppert et al. 2010). The G/G genotype of the variant was found in 14.7 % of subjects compared with only 7.4 % of controls, ($P = 0.005$). The mutation codes for a threonine to alanine substitution in position 17 of the protein (Sherry 2001). This position corresponds to the peptide leader sequence of the CTLA4 receptor. This specific mutation was shown to increase expression of cell-surface CTLA4 receptors on stimulation of T cells, as well as associate with autoimmunity in general (Ligers et al. 2001). These findings further strengthen the interrelatedness of autoimmune disorders and cardiomyopathies as well as present an additional risk marker in DCM pathogenesis.

The Future of nsSNVs and Cardiovascular Diseases

Genomic and Proteomic Projects Worldwide Associated with Cardiac Diseases

There are a high number of institutes and centers worldwide that have recently published papers investigating cardiac systems diseases and conditions through genomic or proteomic means. The high number of international institutes displays that the value of these technologies in identifying potential biomarkers and nsSNV as potential therapeutic and diagnostic targets is globally appreciated. The over 2,000 departments, schools, labs, and centers reinforce the theme of this chapter: namely, that genomic and proteomic technologies are an excellent method of identifying potential therapeutic and diagnostic biomarkers in cardiovascular diseases. In particular, nsSNVs and their associations with cardiovascular disease susceptibility and protection represent value opportunities for further study.

Workflow and Results

Sample Workflow

We present a sample workflow which may be applied to diverse datasets to harness the nsSNVs associated with cardiovascular (or other) diseases as biomarkers. The following workflow was performed for S-nitrosylation but may be repeated and expanded for other features. The importance of S-nitrosylation stems from nitric oxide's role as a relaxation factor derived in the endothelium – where nitric oxide (NO) is largely controlled by S-nitrosylation (Lima et al. 2010). The first step was to

retrieve the human proteome and nine other species (*Mus musculus*, *Bos taurus*, *Canis familiaris*, *Equus caballus*, *Xenopus tropicalis*, *Danio rerio*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Arabidopsis thaliana*) from the UniProtKB/Swiss-Prot database, which is available online.

Next, using the protein BLAST tool, we performed pairwise alignments between the human proteome and nine other species. From the alignment results, all conserved cysteine positions, i.e., the positions which exist in human protein sequences and were mapped at least to one species, were extracted. Cysteine positions were specifically targeted because a cysteine thiol is covalently modified by an NO group to produce S-nitrosothiol (SNO) and thus plays a central role in S-nitrosylation (Lima et al. 2010). Then, the table containing conserved nsSNVs cysteine positions was generated by mapping the conserved cysteine positions among species and human nsSNVs positions from SNVDis (Karagiannis et al. 2013). The GPS-SNO tool (Xue et al. 2010) was used to predict S-nitrosylation sites for the conserved cysteine positions. The rsIDs and swissvarIDs (variation identifier from Swiss-Prot database) obtained from the table of the conserved cysteines and also predicted to be S-nitrosylation sites were used in order to get the information about diseases caused by the variation.

Sample Workflow Results

Results of this workflow are counts, positions, and amino acid variations of observed and predicted disease-related nsSNVs occurring at a conserved cysteine residue of the reference human genome. Please see Tables 1 and 2 for a summary of the results.

Summary Points

- Non-synonymous single-nucleotide variations (nsSNVs) are changes in the genome which ultimately lead to amino acid substitutions and possible changes in biochemical pathways or protein structure or activity.
- Next-generation sequencing (NGS) technologies represent an opportunity to rapidly, cheaply, and efficiently identify nsSNVs as biomarkers and potential therapeutic or diagnostic targets associated with diseases and conditions.

Table 1 Summary counts of impacted proteins, sites, and disease relatedness of genome-wide nsSNV at conserved cysteine residues

	Number of proteins	Number of sites	Diseases
Predicted sites	44	49	84
Experimental sites	7	7	15

This table shows the distribution of nsSNVs among all genomic cysteine positions and the corresponding abundance of potentially affected proteins containing the site and resulting disease relatedness. Although experimental evidence for clinical significance is limited, this omics approach identifies several predicted candidate nsSNVs for further study.

Table 2 Summary of different types of variation and their frequencies

Variation	Predicted sites	Experimental sites
c → r	11	4
c → *	5	
c → y	13	1
c → w	2	
c → s	4	1
c → f	7	1
c → g	6	
c → a	1	

This table shows the distribution of the number of specific amino acid substitutions resulting from nsSNV at a reference cysteine codon. Although several possible substitutions exist, it is interesting to note that only four such substitutions have been observed to date.

- NGS technologies and major software developments have expedited the discovery and analysis of genomic and proteomic data which are essential to the identification of nsSNVs.
- Mapping of the genomic data is facilitating the process of identifying diagnostic and therapeutic targets for a number of diseases and conditions.
- Proteomic approaches can enable cheaper, more rapid, and more robust identification of variation biomarkers and validation of genomic targets against amino acid variants.
- Cardiovascular diseases are an increasing global public health problem, and nsSNVs are playing an integral part in their further study.

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Cardiac Stem Cells as Biomarkers

36

Tiziano Moccetti, Polina Goichberg, Marcello Rota, Annarosa Leri, and Piero Anversa

Contents

Abstract

This chapter discusses the importance that the discovery of resident cardiac stem cells has had and continues to have on our evolving understanding of myocardial biology. Cardiac stem cells have acquired a progressively critical role in myocardial aging and heart failure suggesting that both these processes may be viewed as stem cell diseases. We have focused on the molecular signature and the telomere-telomerase axis to define novel biomarkers able to characterize the growth reserve of the intact and pathologic heart, major determinant of the adaptive and maladaptive response of the myocardium.

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_32

Keywords
Biomarkers • c-kit • Clonal growth • Cell-based therapy • Heart failure

Abbreviations

Abcg2/Bcrp1/Mdr1	ATP-binding cassette transporters, subfamily G
BMPCs	Bone marrow progenitor cells
CHD	Coronary heart disease
c-kit-CPCs	c-kit-positive cardiac progenitor cells
c-kit-CSCs	c-kit-positive cardiac stem cells
CPCs	Cardiac progenitor cells
ECs	Vascular endothelial cells
EF	Ejection fraction
hCPCs	Human cardiac progenitor cells
hCSCs	Human CSCs
LTL	Leukocyte telomere length
MACE	Major adverse cardiac events
MSCs	Mesenchymal stem cells
PDs	Population doublings
Sca1-CPCs	Sca1-positive cardiac progenitor cells
SMCs	Vascular smooth muscle cells
SP	Side population
SP-CPCs	Side population cardiac progenitor cells
Tbx18	T-box18
TIFs	Telomere dysfunction-induced foci
TL	Mean telomere length
WT1	Wilms tumor 1

Key Facts of Cardiac Stem Cells

- The primary function of CSCs is the control of cell turnover and myocardial regeneration.
- The decline in the growth of CSCs is dictated by defects in the telomere-telomerase system.
- The molecular signature of CPC classes has emphasized the critical role that c-kit expression has in defining the undifferentiated cell phenotype.
- Telomere dysfunction has a critical negative impact of CSC behavior and growth.
- Telomere shortening predicts CSC senescence and is an independent predictor of cardiovascular aging and disease.

Definitions

Clonogenicity Clonogenicity corresponds to the ability of individual stem cells to form a cluster of daughter cells identical to the mother cell.

CPCs CPCs are less undifferentiated than CSCs.

CSCs CSCs are a rare population of resident myocardial cells regulating organ homeostasis and repair following injury.

Holoclones, meroclones, and paraclones Holoclones, meroclones, and paraclones indicate, respectively, cell clusters derived from stem cells, early committed cells, and late-stage transit-amplifying cells.

Multipotency Multipotency reflects the ability of stem cells to generate all specialized cell lineages within the tissue.

Self-renewal Self-renewal reflects the ability of stem cells to undergo long-term proliferation and asymmetric division.

Symmetric and asymmetric division Stem cells can divide symmetrically forming two identical daughter cells and asymmetrically generating two differently fated sibling cells.

Telomerase Telomerase is a reverse transcriptase that synthesizes telomeric repeats utilizing its own RNA as a template.

Telomeres Telomeres are double-stranded highly repetitive DNA sequences located at the end of chromosomes.

Whole genome expression profile The global expression profile of cells can be determined by microarray analysis of transcripts.

Introduction

In a well-known editorial, Morrow and de Lemos defined the three criteria that a clinically valuable novel biomarker should meet: (1) the measurement of the biomarker should rely on analytical methods providing the possibility to perform repeated, accurate, and reliable assessments; (2) the biomarker should offer information that cannot be achieved through clinical evaluation; and (3) the ideal biomarker should help in the diagnosis of the disease, the identification of subjects at risk, the monitoring of the response to therapy, and the prognosis of the disease (Morrow and Lemos 2007). Collectively, these parameters constitute the components of the medical decision-making process (Braunwald 2008, 2009).

The majority of biomarkers used in clinical practice do not satisfy the three criteria, but the aggregate evidence provided by clusters of biomarkers typically reveals important variables concerning the etiology, pathophysiology, and/or the progression of the disease (Morrow and Lemos 2007). The advancements in stem cell biology and the implementation of cell-based therapies pose novel challenges

for the search of relevant biomarkers. Stem cell-related biomarkers can be divided in two classes. The first category comprises the identifiers of the phenotype and functional competence of stem cells. This information has to be utilized in the process of quality control of the product, i.e., progenitor cells, prior to administration to patients. Moreover, the evaluation of the phenotypical and functional state of resident stem cells offers evidence related to the residual growth reserve of the organ and, ultimately, may prove or disprove the concept that pathological conditions correspond to stem cell diseases. The second category includes classic biomarkers for the monitoring of the response to cell-based therapy and the tracking of the evolution of the disease.

Multiple classes of stem and progenitor cells have been recognized in the adult myocardium by using surface and intracellular epitopes, functional assays, and their combination (Leri et al. 2015). *Bona fide* adult tissue-specific stem cells are defined by two biomarkers of "stemness": (1) self-renewal, i.e., the ability to divide and form a daughter stem cell that possesses the same properties of the mother cell, and (2) multipotency, i.e., the ability to differentiate in all specialized lineages within a given organ. Optimal biomarkers for the identification and characterization of cardiac progenitor cells (CPCs) comprise membrane antigens, which allow the isolation of the cells from the tissue where they reside and components of signaling pathways involved in the regulation of stem cell growth and commitment.

c-kit-Positive CPCs

More than a decade ago, cells expressing the surface biomarker c-kit were recognized as a population of stem cells residing in the heart of animals and humans (Beltrami et al. 2003; Bearzi et al. 2007). The receptor tyrosine kinase c-kit identifies a pool of CPCs that are self-renewing and multipotent in vitro and in vivo (Beltrami et al. 2003; Angert et al. 2011; Cottage et al. 2012; Fischer et al. 2009; D'Amario et al. 2011; Goichberg et al. 2013; Hariharan et al. 2015). Myocardial cells that possess the properties of stem cells are characterized not only by the presence of c-kit, but also by the absence of bone marrow and cardiac lineage epitopes. A rare population of c-kit-positive CD45-positive bone marrow-derived cells is present in the adult myocardium (Dey et al. 2013; Sanada et al. 2014). These hematopoietic-committed cells may migrate to the heart acquiring temporary or long-term residence. The combination of c-kit and CD45 is also found on the membrane of mast cells, which express inflammatory mediators and proteases, including tryptase and chymase (Sperr et al. 1994). Freshly isolated c-kit-positive CPCs (c-kit-CPCs) are negative for transcription factors and specialized proteins specific for cardiac and vascular fate. The nuclear localization of Nkx2.5, GATA4, and MEF2C and the cytoplasmic distribution of poorly organized sarcomeres recognize early committed c-kit-CPCs that are destined to become cardiomyocytes (Urbanek et al. 2006; Bearzi et al. 2007; Fischer et al. 2009; Hariharan et al. 2015; Sanada et al. 2014). This process of progressive restriction of developmental options (Fig. 1) is equally

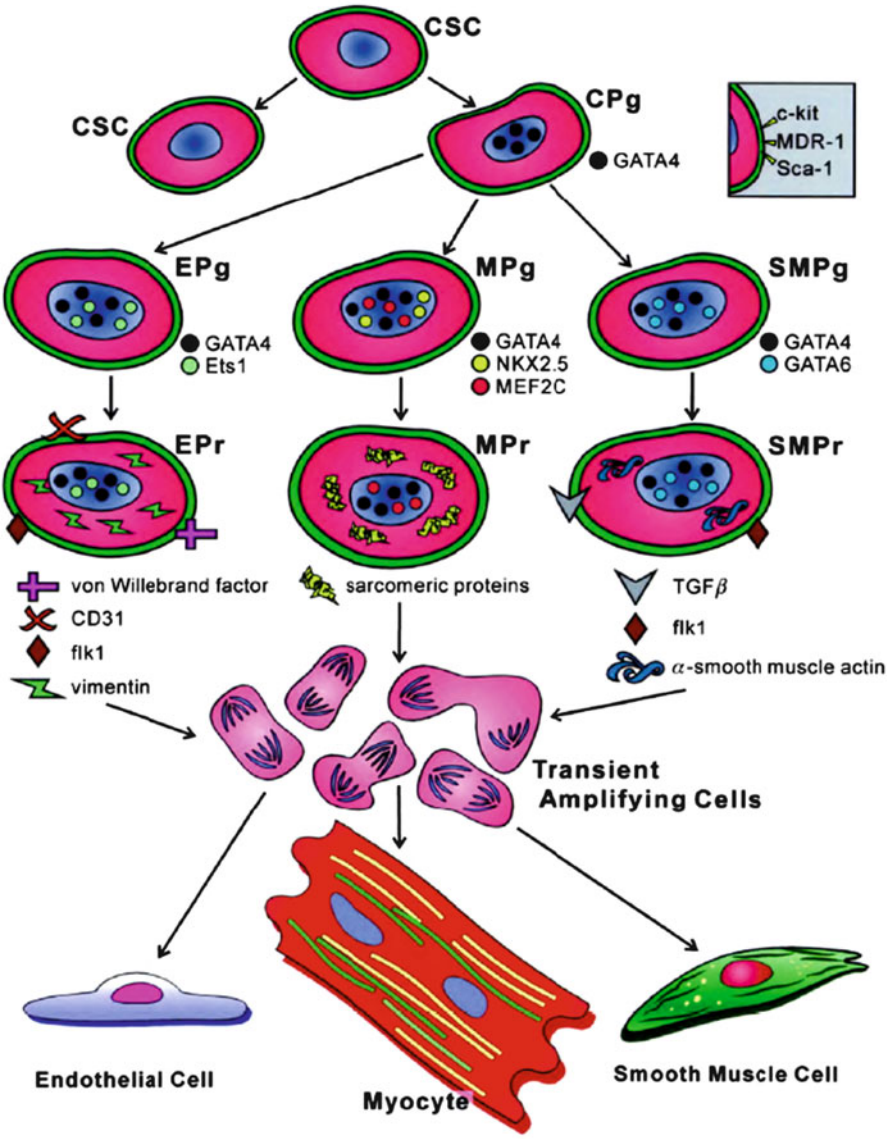


Fig. 1 Cardiac cells are organized hierarchically. Asymmetrical division of a CSC into a daughter CSC and a daughter cardiac progenitor (*CPg*). CPg gives rise to myocyte progenitor (*MPg*) and precursor (*MPr*), EC progenitor (*EPg*) and precursor (*EPr*), and SMC progenitor (*SMPg*) and precursor (*SMPr*). Precursors become transient amplifying cells, which divide and differentiate into mature myocytes, ECs, and SMCs. CSCs are lineage-negative cells that express only c-kit, MDR1, or Sca-1. Progenitors express stem cell antigens and transcription factors of cardiac cells but do not exhibit specific cytoplasmic proteins. Precursors possess stem cell antigens, transcription factors, and membrane and cytoplasmic proteins typical of myocytes, ECs, and SMCs. Amplifying cells have nuclear, cytoplasmic, and membrane proteins of cardiac cell lineages but are negative for stem cell antigens (From Anversa et al. 2006)

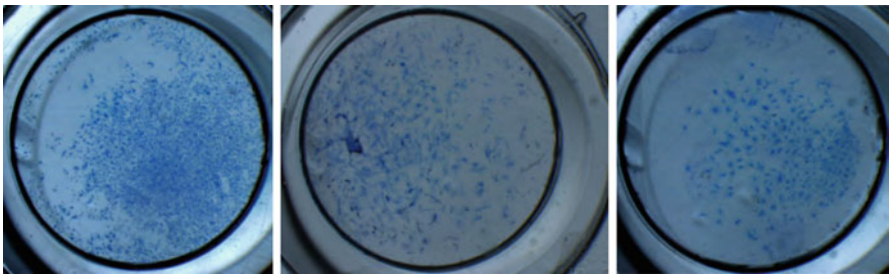


Fig. 2 c-kit-positive cardiac cells form clones with different phenotype. Phase contrast images representative of CSC-derived clones. Clones have different shape and size. Cells are stained with Evans *blue*

operative during the formation of vascular endothelial cells (ECs) and smooth muscle cells (SMCs) and fibroblasts (Anversa et al. 2006).

Lineage-negative c-kit-CPCs undergo serial symmetric divisions in vitro with the formation of daughter stem cells identical to the mother cell (Bearzi et al. 2007; Hariharan et al. 2015). This modality of clonal growth is documented by seeding single c-kit-positive cells in individual wells of Terasaki plates. The characteristics of the clone can be viewed as a biomarker of the functional properties of the parental c-kit-positive cell. Primary cells in culture can form holoclones, meroclones, and paraclones (Jones et al. 2007; Beaver et al. 2014). The fundamental difference among these types of clones is the degree of stemness of the founder cell. Holoclones, which are round in shape and contain a high number of daughter cells, are generated by stem cells capable of undergoing extensive proliferation and self-renewal. Meroclones, which possess an irregular shape, derive from cells with low replicative capacity and self-renewal ability. Paraclones are small in size and are generated by mother cells that have exhausted their growth reserve and rapidly undergo cell cycle withdrawal (Fig. 2). Thus, holoclones, meroclones, and paraclones indicate, respectively, cell clusters derived from stem cells, early committed cells, and late-stage transit-amplifying cells (Jones et al. 2007).

Cardiac c-kit-positive cells form all three types of clones. Large cell clusters that can be expanded to reach a high number of daughter cells are formed by *bona fide* c-kit-positive cardiac stem cells (c-kit-CSCs). In this case, subclones can be obtained upon plating of individual clonal daughter cells (Beltrami et al. 2003). At times, c-kit-positive cardiac cells generate very small colonies, reflecting a stage of early or late commitment; terminal differentiation ensues in a short period of time, indicating that the founder cell has the predicted properties of a precursor (Cesselli et al. 2011). The cells of origin are characterized by low levels of c-kit expression and by the presence of cardiovascular transcription factors and cytoplasmic proteins. These precursors rapidly evolve to the transit-amplifying state giving rise to a small aggregate of cells that are negative for c-kit. These observations underscore the importance of the accurate definition of clones, the thorough characterization of the cell aggregates, and the careful assessment of cloning efficiency. Daughter cells in the stem cell-derived clone preserve their undifferentiated lineage-negative

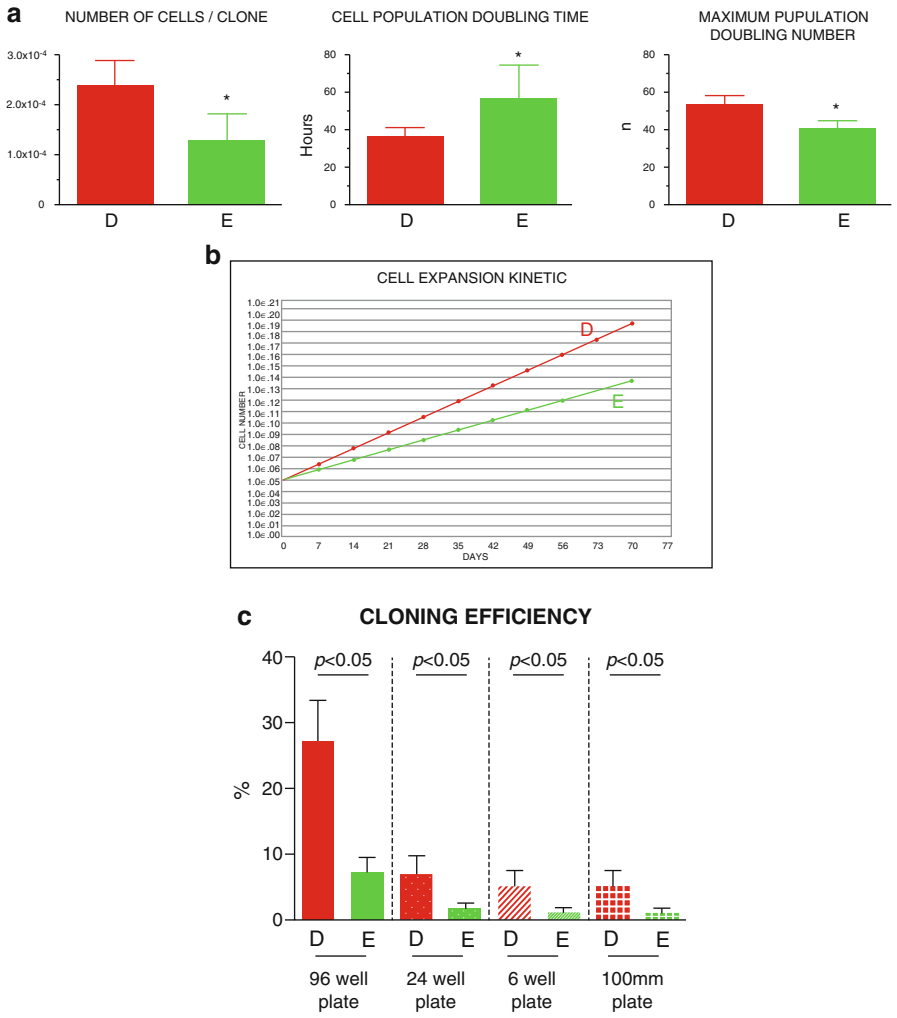


Fig. 3 Human CSCs obtained from control and failing hearts have different functional properties. (**a**) Parameters of cell growth. Results are mean ± SD. *$P < 0.05$ versus donor hearts (*D*). E: Explanted hearts. (**b**) The rate of hCSC expansion is shown in semi-logarithmic scale. (**c**) Cloning efficiency of hCSCs (From Cesselli et al. 2011)

phenotype, continue to express the stem cell antigen, and retain the self-renewal ability and multipotentiality of the founder cell.

To define the effects of disease on CPC clonogenicity, fluorescence activated cell sorting (FACS)-sorted individual c-kit-positive cells were deposited in single wells of 96 multiwell plates (Cesselli et al. 2011). Of 3,682 seeded wells, human CSCs (hCSCs) collected from explanted hearts gave rise to 237 colonies of ~100–200 cells. However, the majority of these small cell clusters underwent growth arrest;

only 39 actively proliferating clones were passaged to 24-well plates where they formed clusters of 40,000–60,000 cells. Nearly 30 % of these colonies stopped growing, while the remaining 70 % were transferred to 6-well plates and continued to replicate undergoing an additional 18 population doublings (PDs) without reaching replicative senescence (Fig. 3a–d). Thus, the actual cloning efficiency of hCSCs from failing hearts was 0.8 % (Fig. 3e). A comparable approach and magnitude of sampling were employed with hCSCs from normal hearts. A cloning efficiency nearly threefold higher was found (Fig. 3e). These clones expanded exponentially for more than 20 PDs (Cesselli et al. 2011).

Evidence of the multipotentiality of c-kit-CSCs can be obtained by exposing clonal cells to differentiation media (Mohsin et al. 2013; Leri et al. 2015). Myocytes, ECs, SMCs, and fibroblasts are formed in various proportions. Typically, CSCs differentiate predominantly into cardiomyocytes and to a lesser extent into ECs and SMCs. Differentiation assays of stem cell clones in vitro have inherent limitations including the possibility that culture conditions result in the preferential acquisition of a selective lineage phenotype, masking the full potential of the founder cell. Similarly, the identification in vivo of multiple cell categories in the progeny of transplanted non-clonal stem cells does not provide direct evidence of the multipotentiality of each delivered cell. This problem can be overcome by the transplantation of clonal populations of CSCs in animal models of the human disease (Beltrami et al. 2003; Bearzi et al. 2007) and by viral gene marking (Hosoda et al. 2009) (Fig. 4).

The adoptive transfer assays have documented that the generation of cardiomyocytes in vivo markedly exceeds the number of cells lost by myocardial infarction. Additionally, a large number of resistance coronary arterioles and a relatively low number of capillary structures develop, resulting in the formation of new myocardial tissue that resembles structurally and functionally the parenchyma of the neonatal heart. The hyperplastic phenotype of the new myocytes and their small size suggest that cardiac repair follows a pattern of growth that reiterates the expected evolutionary changes coupled with the activation of a stem cell (Leri et al. 2015). The reconstitution of myocardium within the necrotic or scarred region reduces proportionally the extent of damage, reversing partly ventricular dilation and thinning of the wall. However, the normal architecture and orientation of myocyte bundles across the wall are not typically acquired.

In the majority of cases, short intervals after c-kit-CSC treatment have been studied, raising the critical question whether young myocytes could become fully mature adult cells with time. This process, which requires a significant increase in cell volume with the insertion of new myofibrils occupying the expanding cytoplasm, may be time dependent. In the acute phases of cardiac repair, in situ activation of resident c-kit-CPCs results in the commitment to the myocyte lineage and formation of small amplifying myocytes with a volume of ~2,000 μm^3 (Urbanek et al. 2005). However, 4 months later, myocyte size averaged ~5,000 μm^3, and nearly 10 % of myocytes reached the adult phenotype, 10,000 μm^3 in volume or larger.

In all organs, stem cells are relatively rare; the frequency of c-kit-CPCs in animals and humans, 1 every 30,000–40,000 myocardial cells (Beltrami et al. 2003; Urbanek et al. 2005; Bearzi et al. 2007), is consistent with that of hematopoietic stem cells in

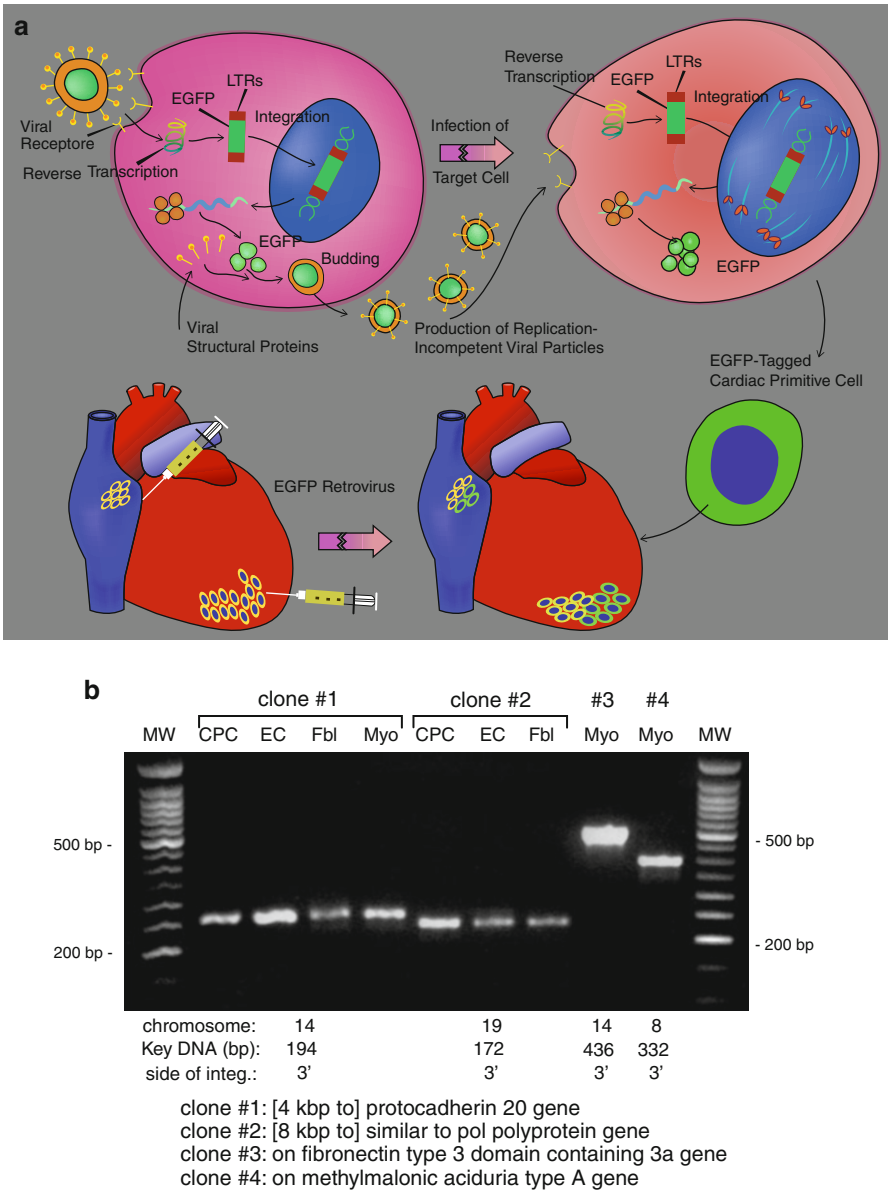


Fig. 4 The self-renewal ability, clonogenicity, and multipotency of CPCs in situ were documented by viral genetic tagging. (**a**) Schematic representation of the protocol employed for clonal marking of mouse CPCs in situ. Infection of CPCs in situ with EGFP retroviruses or EGFP lentiviruses results in the semi-random insertion of the proviral integrant in the genome of the recipient cell. Transcription and translation of the viral DNA result in expression of EGFP and fluorescent labeling of the infected CPC. The unique insertion site of the viral genome is inherited by the entire population derived from the parental cell and can be amplified by PCR. CPCs nested in

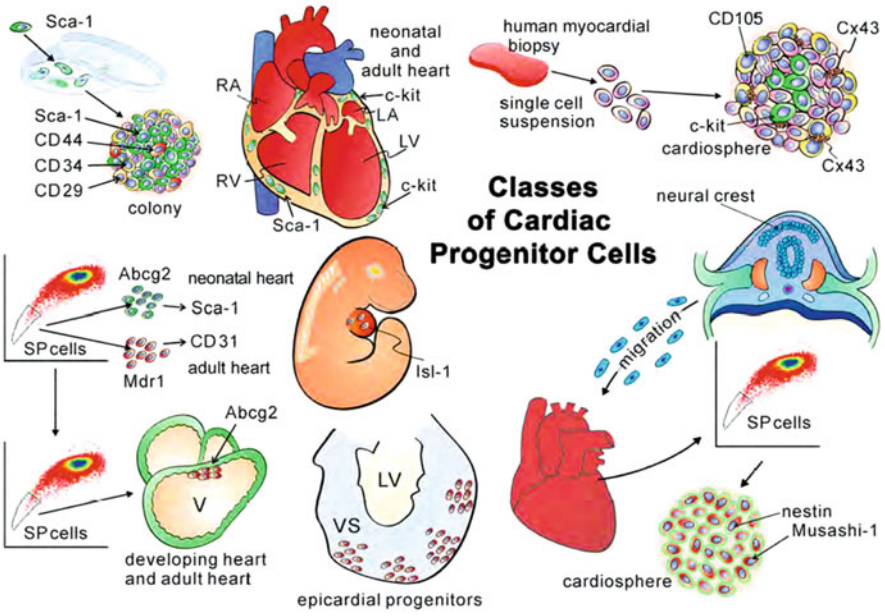


Fig. 5 **The developing and adult heart contains multiple classes of CPCs**. Schematic representation of CPC populations

the bone marrow, 1 every 10,000–100,000 (Shepherd et al. 2007). c-kit-CPCs are present in the entire ventricular myocardium, but are preferentially distributed to the atria and apex (Urbanek et al. 2006). The adult heart typically shows interstitial structures with the architectural organization of stem cell niches. c-kit-CPCs are functionally coupled to myocytes and fibroblasts by adherens junctions expressing N- and E-cadherins and by gap junctions expressing connexin 43 and 45 (Leri et al. 2014). Because of this anatomical configuration, myocytes and fibroblasts operate as supporting cells within the cardiac niches, which provide the necessary permissive milieu for the long-term residence, survival, and growth of CPCs. The function of cardiomyocytes as modulators of c-kit-CPC growth and differentiation has been documented in vitro and in vivo.

Fig. 4 (continued) atrial and apical niches were labeled in situ to identify their progeny in vivo. One to 6 months later. The heart was enzymatically dissociated to obtain cardiomyocytes, c-kit-CPCs, ECs, and fibroblasts. (**b**) Four distinct clones were identified in EGFP-tagged CPCs, ECs, fibroblasts (*Fbl*), and cardiomyocytes (*Myo*) isolated from the ventricle of one mouse heart. Multiple PCR products (bands in agarose gel) were identified. Bands of the same molecular weight correspond to identical sites of integration of the proviral sequence in the host genome of CPCs, myocytes, ECs, and fibroblasts, documenting the multipotency of CSCs in vivo

Myocardial Progenitors

Different classes of progenitor cells have been characterized in the adult heart, but whether they represent distinct categories of undifferentiated cells with diverse functional properties is currently unknown (Fig. 5). The first identification of myocardial progenitors was based on the ability of stem cells to expel toxic compounds and dyes through an ATP-binding cassette transporter (Hierlihy et al. 2002). This property, used initially to isolate side population (SP) hematopoietic cells (Challen and Little 2006), defines a pool of putative cardiac progenitors that form colonies in semisolid media and differentiate into cardiomyocytes. Depletion of cardiac SP cells occurs after infarction in mice overexpressing a dominant-negative MEF2C, documenting that SP cells are committed to the myocyte lineage. This work introduced the concept of a myocardial stem cell that participates in the response of the heart to ischemic injury (Hierlihy et al. 2002).

Evidence of ABC-transporter activity can be obtained by exposing cardiac cells to the DNA-binding dye Hoechst 33342; functionally competent cells clear the fluorochrome, become Hoechst-low, and occupy a side position in the FACS profile (Pfister et al. 2010; Lin and Goodell 2011). SP cells, which are $Sca1^{high}$, $c\text{-}kit^{low}$, $CD34^{low}$, and $CD45^{low}$, comprise 2 % of cardiac cells in the mouse heart. The ability to extrude dyes is attributed to the expression of the multidrug resistance protein Abcg2/Bcrp1 (Martin et al. 2004, 2008). However, most Bcrp1-positive cells express CD31 and are located within the intima of the vessel wall. After injury, SP cells generate predominantly vimentin-positive fibroblasts and calponin-positive SMCs; only few cells acquire the myocyte and EC lineage. A subset of SP cells expresses markers of neural precursors, including nestin and Musashi-1; they form in vitro neuron-like dendrites and peripheral nerve cells (Tomita et al. 2005). These SP cells correspond to embryonic fetal remnants of neural crest-derived cells.

By introducing a novel protocol, an interesting class of SP cardiac progenitor cells (SP-CPCs) was identified (Sereti et al. 2013). Only the Sca1-positive CD31-negative subset of SP-CPCs retained a high cardiomyogenic potential (Pfister et al. 2005). Abcg2 promotes proliferation and survival of SP cells inhibiting differentiation (Sereti et al. 2013). Dysregulation of Abcg2 may alter the fate of these progenitors, resulting in uncontrolled cell growth or death. Bone marrow SP cells do not contribute to the maintenance of the SP-CPCs in physiological conditions, but can repopulate the resident pool after injury (Mouquet et al. 2005).

State-of-the-art imaging protocols have been developed to track in vivo the destiny of Sca1-positive cardiac progenitor cells (Sca1-CPCs). These cells were isolated from transgenic mice that constitutively express luciferase and enhanced green fluorescent protein (EGFP), enabling in vivo tracking by noninvasive imaging and postmortem identification by immunolabeling (Li et al. 2009; Swijnenburg et al. 2010). A strong bioluminescence signal was detected at 2 days after cell delivery; however, this signal decayed rapidly with time. The nonviable portion of the ventricular wall, which was studied by (18F)-FDG positron emission tomography scan, was not reduced in cell-treated mice. Consistently, echocardiographic and MRI analyses did not show functional improvement, and a very small number of

EGFP-positive cardiomyocytes and vascular structures were found. Poor survival and massive apoptosis of the delivered cells are commonly observed when neonatal cardiomyocytes, mesenchymal stem cells (MSCs), bone marrow mononuclear cells, and human embryonic stem cell-derived cardiomyocytes are adoptively transferred (Dowell et al. 2003; Leri et al. 2005; Laflamme et al. 2007; Robey et al. 2008; Wiliams and Hare 2011; Quijada et al. 2012; Siddiqi and Sussman 2013). This phenomenon has prompted the development of novel strategies involving preactivation with growth factors, application of bioengineering methods, and genetic modifications to achieve long-term homing to the injured myocardium (Laflamme et al. 2007; Tillmanns et al. 2008; Siddiqi and Sussman 2013). Collectively, the results with SP cells and Sca1-CPCs suggest that a small pool of primitive cells, distinct from c-kit-positive CPCs, is present in the myocardium. Genetic deletion of Sca1 alters the function of resident c-kit-CPCs, leading to premature alterations in cardiac performance and poor tolerance of the heart to stress (Bailey et al. 2012).

An alternative source of progenitor cells has been found in the epicardium, which represents an epithelial sheet on the cardiac surface. The epicardial marker Wt1 regulates the epithelial-mesenchymal transition (von Gise et al. 2011). Wt1-positive progenitors travel from the proepicardium to the myocardium where they form the epicardium and electrically coupled cardiomyocytes (Zhou et al. 2008). Moreover, a population of proepicardial Tbx18-positive progenitors may give rise to a substantial fraction of cardiomyocytes (Cai et al. 2008). A recent study has challenged the initial findings obtained with Wt1-based Cre/LoxP strategy (Rudat and Kispert 2012). Limitations have been identified in the lineage-tracing mouse employed. They include the poor recombination efficiency of Wt1-positive cells, the lack of specificity of the promoter due to the endogenous extra-epicardial localization of Wt1 in ECs, and the possibility of ectopic recombination (Rudat and Kispert 2012; Zhou and Pu 2012).

A pool of c-kit-positive epicardial cells has been identified in the human infarcted heart (Castaldo et al. 2008). Experimentally, c-kit-positive epicardial cells migrate from the epicardium to the infarcted myocardium, where they proliferate and differentiate into myocyte precursors and vascular cells (Limana et al. 2007). This process is coupled with upregulation of fetal epicardial markers. The recognition of growth factors modulating the behavior of epicardial progenitors may allow their in situ activation, possibly influencing the treatment of the human disease.

Expansion of cardiac cells from human endomyocardial biopsies leads to the formation of floating spheres. Cardiospheres contain a small core of c-kit-positive primitive cells, several layers of differentiating cells expressing myocyte proteins and connexin 43, and an outer sheet composed of MSCs (Smith et al. 2007; Davis et al. 2010; Lee et al. 2011). c-kit-positive cells within the aggregates do not correspond to a uniform class of progenitors because of their heterogeneity dictated by the uncommitted or early committed state, their quiescent or cycling condition, and their migratory properties. This may explain the observed differences in the regenerative potential of single-cell-derived clonal c-kit-CSCs (Beltrami et al. 2003) and c-kit-positive cells sorted from cardiospheres (Cheng et al. 2014).

In summary, different progenitor cell categories may participate in myocardial homeostasis and repair after damage. The surface antigens, transcription factors, and functional assays discussed above constitute novel biomarkers that allow the characterization of resident myocardial progenitors. This information may provide important insights on the etiology, pathophysiology, and evolution of cardiac diseases. Currently, two distinct classes of cardiac-derived cells have been tested clinically, c-kit-CPCs and cardiosphere-derived cells (Bolli et al. 2011; Makkar et al. 2012). The phase 1 trial SCIPIO (Stem Cell Infusion in Patients with Ischemic cardiOmyopathy) involved the delivery of autologous c-kit-positive human CPCs (hCPCs) for the treatment of severe chronic heart failure of ischemic origin (Bolli et al. 2011). Patients with ejection fraction (EF) lower than 40 % at 4 months after coronary artery bypass grafting were enrolled in the treatment and control groups. Treated patients received a single intracoronary infusion of one million autologous hCPCs. Importantly, no adverse effects were reported in the 14 patients treated with hCPCs. EF increased from 30 % to 38 % at 4 months after infusion. The beneficial effects of CPCs were even more pronounced at 1 year and remained stable thereafter. In treated patients, infarct size decreased 24 % and 30 % at 4 and 12 months, respectively.

The prospective, randomized CADUCEUS (CArdiosphere-Derived aUtologous stem CElls to reverse ventricUlar dySfunction) trial included patients with subacute myocardial infarction and 25–45 % EF (Makkar et al. 2012). Autologous cells grown from endomyocardial biopsy specimens were infused into the infarct-related artery 1.5–3 months after infarction. The primary endpoint consisted of the proportion of patients at 6 months who died due to arrhythmic events or had myocardial infarction after cell infusion, new cardiac tumor, or a major adverse cardiac event (MACE). Additionally, preliminary data concerning the efficacy of the treatment were collected by MRI at 6 months. At baseline, mean EF was 39 % and the scar occupied 24 % of the left ventricular mass. At 6 months, no patients had died, developed cardiac tumors, or MACE in either group. However, four patients in the cell-treated group had serious adverse events compared with one control. Cell therapy resulted in a reduction in scar mass, increase in viable heart mass, and enhanced regional contractility and regional systolic wall thickening. End-diastolic volume, end-systolic volume, and EF did not differ between treated and untreated groups. The initial results of SCIPIO and CADUCEUS trials are highly encouraging and warrant further, larger, phase 2 studies.

Molecular Signature of CPCs

The discovery of multiple CPC classes in the adult myocardium is intriguing, but it may not be surprising if we consider other stem cell-regulated tissues. The implementation of complementary strategies has led to the identification of distinct pools of progenitor cells in several organs, including the small intestine, the skin, and the brain. Side-by-side comparisons of the characteristics of different CPC types have rarely been performed, leaving unanswered the question whether the several cell

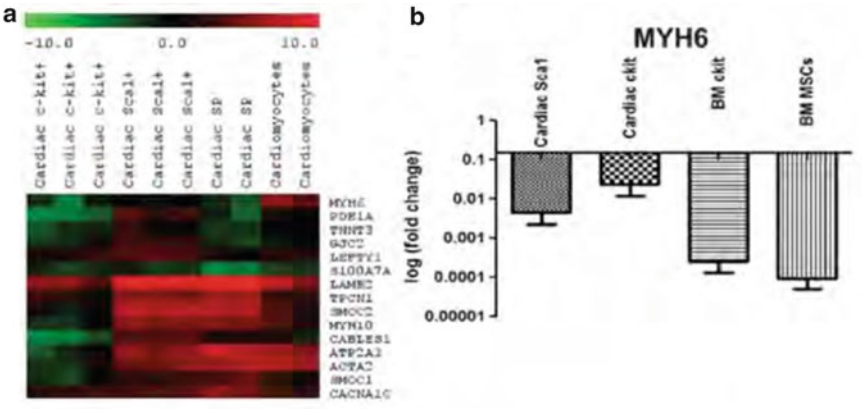
populations identified in the adult heart correspond to different stages of maturation of the same parental cell. In the search for novel biomarkers specific for distinct CPC populations, the molecular signature of c-kit-CPCs, Sca1-CPCs, and SP-CPCs was studied using whole genome transcriptional profiling. The molecular footprint of CPCs was compared with that of cardiomyocytes, bone marrow c-kit-positive progenitor cells (BMPCs), and MSCs.

A significant expression difference of 1,438 genes was found among the three classes of CPCs and between CPCs and cardiomyocytes. The global expression of genes characteristic of the terminally differentiated state of cardiomyocytes was significantly downregulated in the three CPC populations. However, the extent of commitment of CPCs to the myocyte fate differed in the three cell pools: Sca1-CPCs showed the highest correlation with cardiomyocytes, SP-CPCs an intermediate value, and c-kit-CPCs the lowest (Fig. 6a–c). Myocyte-restricted transcription factors, sarcomeric proteins, ion transporters, and calcium-binding proteins were upregulated in Sca1-CPCs and SP-CPCs (Dey et al. 2013).

These findings indicate that early myocyte-lineage genes are poised for expression in Sca1-CPCs and SP-CPCs. Mef2c, Nkx2.5, Tbx5, and Gata4 constitute the critical core of transcription factors that regulate cardiomyogenesis in the embryonic and fetal heart. Similarly, Kv2.2 isoform mRNA is highly enriched in Sca1-CPCs and SP-CPCs. This potassium channel subunit is typically present in embryonic myocytes at very early stages of development and may correspond to the immature equivalent of the protein in the adult organ. Expression of the skeletal and smooth muscle genes Tnnt3 and Acta2 in Sca1-CPCs and SP-CPCs is also indicative of an early state of myocyte lineage determination of the two cell subsets. Collectively, the expression of genes of early myocyte commitment in Sca1-CPCs and SP-CPCs is consistent with the view that myocyte renewal in the adult heart recapitulates cardiac morphogenesis in prenatal life. By comparative analysis and hierarchical clustering, the transcriptional profile of c-kit-CPCs reflects a highly undifferentiated phenotype (Fig. 6d).

The chromatin structure predictive of the multipotent state of c-kit-CPCs may carry a bivalent conformation of histones characterized by activating and inactivating modifications in the same or adjacent nucleosomes. In multipotent progenitor cells, genes that are required in the differentiated progeny are transiently held in a repressed state by histone modifications, while genes that are associated with stemness are stably maintained in an active state. With differentiation, genes that are crucial for multipotency are silenced through histone modifications and DNA methylation. The acquisition of a specific lineage imposes the upregulation of a selected network of genes and the silencing of all other differentiation programs within the cells.

Early mesoderm genes and genes involved in the Notch and canonical Wnt signaling pathways were upregulated in c-kit-CPCs (Fig. 7a). Enzymes controlling cell signaling and metabolism, ATP-binding cassette transporters, anionic transporters, chemokines, and interleukin receptors showed enriched expression in c-kit-CPCs (Fig. 7b, c). The main downregulated core of genes in c-kit-CPCs comprised genes encoding for extracellular matrix proteins, integrins, matrix metalloproteases, and gap junctions (Fig. 7d). Additionally, transcripts for



c

	Compared to Sca1$^+$ cells		Compared to SP cells	
Gene name	**(log)2 ratio**	**p value**	**(log)2 ratio**	**p value**
MEF2C	-7.90827	0.000621	-6.33012	0.006722
KCNB2	-1.45793	0.004817	-2.40072	0.003199
MYH10	-6.54908	0.000693	-6.71755	0.004894
SMOC2	-7.14649	0.000497	-7.05311	0.007834
SLC9A9	-7.63601	0.003585	-5.13441	0.04474
HAND2	-5.24312	0.015957	-7.32604	0.023708
HEG1	-2.55583	0.017103	-2.5681	0.042973
RORA	-2.41246	0.009546	-3.16497	0.018995

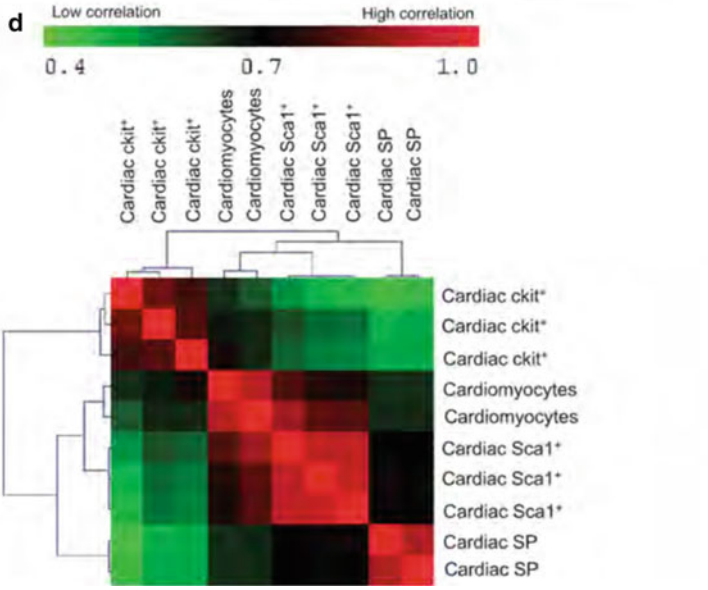


Fig. 6 **Transcriptional profile of cardiac-derived cells**. (**a**) Heat map representing hierarchical clustering of cardiac and/or muscle-specific genes. (**b**) Transcripts for α-myosin heavy chain (*MYH6*). Results are shown as fold changes in mRNA level with respect to cardiomyocytes. (**c**) Myocardial-restricted genes upregulated in Sca1-CPCs and SP-CPCs with respect to c-kit-CPCs.

biomarkers of vascular ECs and fibroblasts and genes involved in connective tissue remodeling were downregulated in c-kit-CPCs. These findings confirm that the transcriptional profiling of c-kit-CPCs differs significantly from that of Sca1-CPCs and SP-CPCs (Dey et al. 2013).

Despite the shared expression of the c-kit receptor tyrosine kinase, c-kit-BMPCs and c-kit-CPCs showed a highly distinct molecular signature (Fig. 8a). The upregulation of a large gene network involved in DNA replication, repair, and cell cycle regulation in c-kit-BMPCs is consistent with the rapid turnover of the hematopoietic system. Conversely, 40–60 % of c-kit-CPCs are quiescent in the young and old myocardium. Thus, in healthy organisms, the level of expression of genes involved in DNA damage response and cell division appears to represent a cluster of biomarkers able to distinguish c-kit-positive progenitor cells with long-term residence in the myocardium and c-kit-positive cells, which migrate from the bone marrow to the heart (Fig. 8b).

These data document that c-kit-CPCs, Sca1-CPCs, and SP-CPCs represent three distinct cell populations at different stages of commitment. However, the demonstration whether Sca1-CPCs and SP-CPCs originate from c-kit-CPC requires carefully designed lineage-tracing studies in vivo. The findings discussed thus far do not provide information concerning the effects of age and disease on human c-kit-CPCs. The transcriptional profile of human c-kit-CPCs obtained from the atria of donor and explanted hearts was conducted by microarray analysis (Cesselli et al. 2011). Transcripts of genes involved in wound healing and response to stress were upregulated in hCSCs from explanted hearts. Similarly, a group of chemokines and inflammatory factors were more represented in these stem cells, possibly reflecting their proficiency to engraft within the microenvironment of the failing heart. The expression of a subset of genes implicated in lipid metabolism was decreased in hCPCs from failing hearts, suggesting a metabolic shift from fatty acid to carbohydrate metabolism, together with a reduction in mitochondrial-encoded gene expression and protein synthesis. An attenuated response of genes involved in the adaptation to oxidative stress was also observed (Fig. 9).

Highly differentially expressed genes in hCPCs from donor and explanted hearts were then analyzed using Ingenuity Pathway Analysis Software. This independent approach highlighted the presence of two functional gene cores differentially represented in the two classes of hCPCs. The first core included the components of the molecular systems that regulate cell growth, proliferation, motility, lipid and carbohydrate metabolism, and cell-to-cell signaling. The second core involved genes implicated in cardiac development, homeostasis, and repair. Molecular targets of known therapeutic agents used in the treatment of cardiac diseases were upregulated

Fig. 6 (continued) (**d**) Correlation among the three cardiac-derived CPCs and cardiomyocytes represented as a hierarchical cluster matrix, based on Pearson's correlation of significant ($p < 0.05$) differentially expressed genes (≥2-fold) among all samples. *Red* represents high correlation; *green* represents low correlation (From Dey et al. 2013)

Early developmental genes, mesodermal-specific markers and stem cell signaling molecules upregulated in cardiac ckit$^+$ cells with respect to cardiac Sca1$^+$ and SP cells

	Compared to Sca1$^+$ cells		Compared to SP cells	
Gene name	**(log)2 ratio**	**p value**	**(log)2 ratio**	**p value**
NKX2-3	2.947868	0.006842	2.315157	0.022146
TBX1	3.258979	0.002865	1.880969	0.044091
MEST	4.596251	0.030854	6.397683	0.040677
EDNRB	5.880017	0.009592	5.555687	0.033576
Fzd10	1.506756	0.035476	2.121195	0.030591
DLL4	3.793231	0.021916	4.900770	0.014582
LRP8	5.047116	0.002544	3.812955	0.004395
ERN1	3.293769	0.030332	4.990999	0.043222
SOX18	5.022194	0.043175	6.245570	0.005383
ASPSCR1	1.655397	0.004993	1.650760	0.024680
IGHMBP2	1.515657	0.031217	1.255008	0.047935

Genes encoding kinases and metabolic pathway molecules upregulated in cardiac ckit$^+$ cells with respect to cardiac Sca1$^+$ and SP cells

	Compared to Sca1$^+$ cells		Compared to SP cells	
Gene name	**(log)2 ratio**	**p value**	**(log)2 ratio**	**p value**
MYLK2	2.449544	0.033229	2.459230	0.018207
GCLM	4.914408	0.001751	4.731067	0.018651
GSR	3.404869	0.015610	3.704986	0.035554
CHST4	2.989557	0.004743	2.096534	0.042825
HPGDS	3.826584	0.037901	2.244285	0.026049
INPP4A	4.294689	0.002351	3.124071	0.026109
PIK3CG	2.281688	0.034717	3.170903	0.034520
NQO1	6.992044	0.004179	6.124394	0.033839
ATP7B	1.275695	0.001582	7.361509	0.000859
ADH7	3.046368	0.025319	2.518860	0.022582
PHLDA2	5.328909	0.023177	7.249410	0.029028

Genes encoding transporters and ion channels which are upregulated in cardiac ckit$^+$ cells with respect to cardiac Sca1$^+$ and SP cells

	Compared to Sca1$^+$ cells		Compared to SP cells	
Gene name	**(log)2 ratio**	**p value**	**(log)2 ratio**	**p value**
ABCG5	1.489033	0.040843	1.412719	0.031027
SLC7A11	5.855062	0.002628	6.779846	0.011295
SLC25A13	2.666909	0.007270	2.044830	0.035511
KCNJ2	5.562056	0.046619	6.318745	0.022832
OTOP1	2.597527	0.025956	3.157605	0.021380

Chemokines, interleukin receptors, and hematopoietic cell-specific genes upregulated in cardiac ckit$^+$ cells with respect to cardiac Sca1$^+$ and SP cells

	Compared to Sca1$^+$ cells		Compared to SP cells	
Gene name	**(log)2 ratio**	**p value**	**(log)2 ratio**	**p value**
CMA1	5.451747	0.006161	4.940153	0.009952
CSF2RB	4.919763	0.026923	6.292858	0.027126
HHEX	5.982009	0.046852	6.403108	0.009518
SRGN	2.927116	0.040110	6.508746	0.008196
SLPI	7.372099	0.010182	9.403814	0.007264
IL7R	6.913959	0.006677	4.000399	0.033862
CCL22	3.912088	4.46E-05	2.949126	0.001592
TNFRSF18	3.050739	0.005701	2.696391	0.041761
ST8SIA4	5.269708	0.024284	9.390443	0.011375
AIRE	1.692580	0.030879	2.930811	0.007024
TPSB2	3.911608	0.002290	3.933493	0.007794
ILIF10	2.634135	0.021475	3.133199	0.015748

Fig. 7 Gene enrichment in c-kit-CPCs. (**a**–**d**) Genes pertaining to four functional categories were upregulated in c-kit-CPCs versus Sca1-CPCs and SP-CPCs (From Dey et al. 2013)

in hCPCs from decompensated hearts. Thus, the molecular identity of hCPCs from failing hearts differs substantially from that of hCPCs from control myocardium, providing potential biomarkers of their growth and regeneration capacity.

Telomere Length and Cardiovascular Diseases

Telomeres are composed of double-stranded TTAGGG repeats that encompass 9–15 kb in humans. Telomere length is partly maintained by telomerase, a specialized ribonucleoprotein that adds telomeric DNA at the end of chromosomes. Epidemiologic evidence suggests that shortening of mean telomere length (TL) in white blood cells is correlated with cardiac and vascular pathologies (Spyridopoulos and Dimmeler 2007; De Meyer et al. 2011). However, not all studies are in agreement, and the relevance of telomere length as biomarker of cardiac disease and aging has been questioned (Hoffmann and Spyridopoulos 2011). Telomere dysfunction has been implicated in aging and senescence, and shorter TL in peripheral blood cells predicts cardiovascular disease and mortality. Numerous factors have prevented its broad use as a surrogate endpoint; they include the type and stage of the disease, the

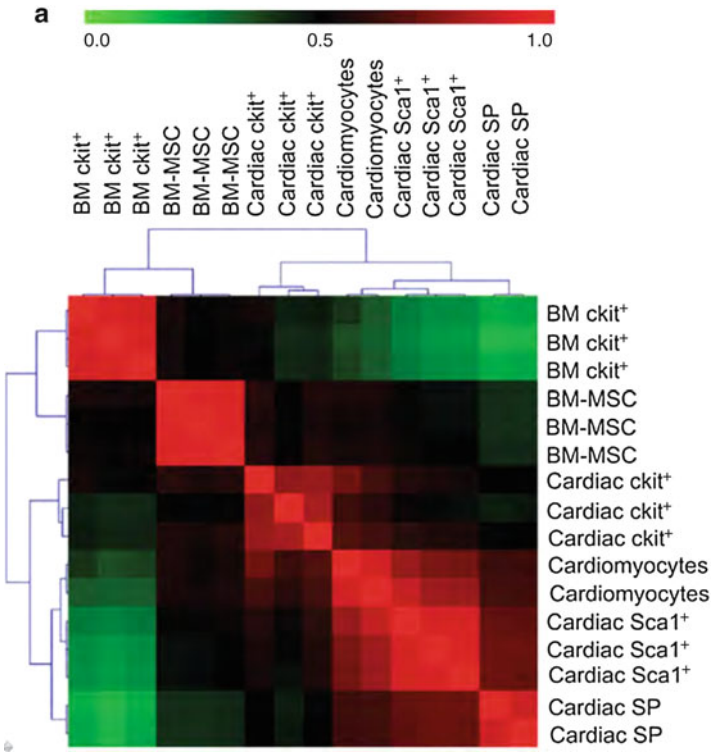


Fig. 8 (continued)

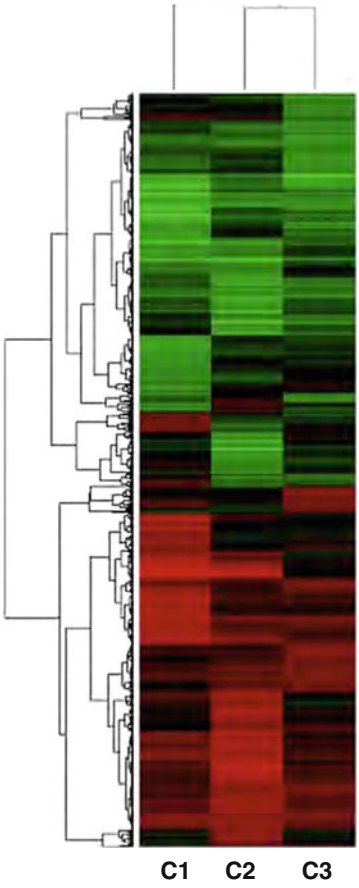
b

Genes downregulated in cardiac cells in comparison to BM cells: Cell cycle and DNA repair

	Compared to BM cells			Compared to BM cells	
Gene name	**(log)2 ratio**	**p value**	**Gene name**	**(log)2 ratio**	**p value**
RAD51	-6.65702	3.81E-05	CCNA2	-8.64993	2.58E-06
RAD51AP1	-2.93829	0.004609	NEK2	-6.43639	1.93E-05
RAD54L	-1.61247	0.017775	NUSAP1	-3.17454	0.005471
DCLRE1B	-3.51313	0.001809	PSRC1	-3.20433	5.57E-05
MCM10	-6.1459	1.13E-05	PSMC3IP	-4.99759	0.001038
MCM7	-1.6482	0.024817	FOXM1	-6.27482	2.69E-05
ZWILCH	-4.14524	3.7E-05	LSM11	-2.26321	5.66E-06
CDKN3	-5.91979	1.01E-05	CKAP2	-4.51619	6.97E-06
CKS2	-1.99491	0.022946	CLSPN	-3.6339	1.89E-05
TOP2A	-5.89237	7.81E-05	MKI67	-6.82292	3.69E-09
PLK1	-5.30944	1.96E-08	CENPA	-7.73735	4.31E-05
NCAPG2	-4.20411	0.001383	CENPH	-7.51336	2.06E-08
NCAPD2	-3.38608	1.77E-05	PMS2	-2.28623	0.034174
BUB1	-8.51468	2.31E-08	SMC2	-3.1528	0.000551
KIF22	-4.98415	4.73E-08	RRM1	-2.30607	0.004292
KIF15	-6.66958	1.4E-06	CENPE	-4.5074	1.85E-05
KIF20B	-3.38301	0.001008	CDCA8	-4.9866	0.000151
KIF23	-5.3509	2.06E-05	CDCA5	-3.95208	3.59E-05
TPX2	-5.39631	0.001477	CDC25C	-5.3415	1.45E-05
POLA1	-4.11884	0.001609	GMNN	-6.20988	6.41E-06
MAD2L1	-6.13612	4.39E-05	CIT	-5.19111	0.000299
MASTL	-2.61873	0.00051	UHRF1	-6.17743	0.000559
HAUS4	-2.8222	0.017249	POLA1	-4.11884	0.001609
BUB1	-8.51468	2.31E-08	PNKP	-5.61481	0.000371
SEPT9	-2.61778	0.000299	TTK	-5.60226	1.62E-05
PMF1	-2.16669	0.002	EXO1	-5.38878	1.43E-05
E2F8	-6.59104	4.49E-06	HELLS	-3.17076	0.000423
E2F2	-6.01447	9.7E-07	FBXO5	-3.78804	3.34E-07
DBF4	-4.43398	0.003481	POLE	-4.13327	0.035291
DTL	-4.17687	1.79E-05	XPC	-2.58405	0.035842
GSG2	-5.47065	4.05E-06	RECQL5	-2.40987	0.011819
SKA1	-1.62069	0.007228	DNA2	-5.85404	0.000402
BRIP1	-1.65905	0.000102	NEIL3	-8.59313	1.06E-08
NDC80	-6.07966	0.000156	CCN5	-8.08324	1.73E-05
CCDC88B	-7.37681	1.42E-07	POLQ	-7.21789	4.2E-08
CASC5	-7.35892	2.42E-06	GAS2L3	-7.04776	1.98E-07
MKI67	-6.82292	3.69E-09			

Fig. 8 Hierarchical clustering among cardiac-derived and bone marrow-derived cells. (**a**) Correlation among the three cardiac-derived CPCs, cardiomyocytes, and BM-derived cells, represented as a hierarchical cluster matrix, based on Pearson's correlation test of significant ($p < 0.05$) differentially expressed genes (≥2-fold) among all samples. *Red* represents high correlation; *green* represents poor correlation. (**b**) A subset of genes involved in DNA repair response and cell cycle is enriched in c-kit-BMPCs with respect to CPCs (From Dey et al. 2013)

age and gender of healthy individuals and patients, the methodology used for the measurement of TL, and the peripheral blood cell population in which TL is assessed.



GENES UPREGULATED IN hCSCs FROM EXPLANTED HEARTS

SYMBOL	FOLD CHANGE	P-VALUE	FAMILY
IL6	11.54	0.0002	CYTOKINE
IL1B	8.23	0.0001	CYTOKINE
ADRA2A	2.87	0.0497	G-PROTEIN COUPLED RECEPTOR
ACE	2.85	0.0006	PEPTIDASE
IGFBP7	2.61	0.0004	TRANSPORTER
CXCL6	2.39	0.0005	CYTOKINE
ADRB2	2.38	0.0158	G-PROTEIN COUPLED RECEPTOR
CX3CL1	2.21	0.0006	CYTOKINE
LIF	1.89	0.0161	CYTOKINE
TGFBR2	1.86	0.0026	KINASE
AKT1	1.84	0.0061	KINASE

GENES UPREGULATED IN hCSCs FROM DONOR HEARTS

SYMBOL	FOLD CHANGE	P-VALUE	FAMILY
KCNA5	4.12	0.0327	ION CHANNEL
NKX2-5	3.81	0.0275	TRANSCRIPTION REGULATOR
KCNMB4	2.45	0.0209	ION CHANNEL
IRS2	2.35	0.0064	OTHER
KCNMB2	2.33	0.0478	ION CHANNEL
FLT1	2.24	0.0433	KINASE
KCNMB1	2.24	0.049	ION CHANNEL
KCNH2	2.17	0.0329	ION CHANNEL
ADRBK2	2.05	0.0358	KINASE
COLIA1	2.05	0.0001	OTHER
ADRAID	2.05	0.4783	G-PROTEIN COUPLED RECEPTOR
CCL1	1.98	0.0406	CYTOKINE
NOS2A	1.89	0.0308	ENZYME

Fig. 9 Transcriptional profile of hCPCs. Heat map showing the differentially expressed genes in hCPCs from donor and explanted hearts. Each column (*C1*, *C2*, and *C3*) represents the direct comparison of hCSCs collected from age- and sex-matched donor and failing hearts. C1, C2, and C3 reflect three different sex- and age-matched pairs. Genes downregulated and upregulated in hCSCs from donor hearts are shown in *green* and *red*, respectively. Fold changes are shown for a selected list of genes (From Cesselli et al. 2011)

Although leukocyte telomere length (LTL) has been associated with a variety of aging-related cardiovascular diseases, recent studies have emphasized the relevance of specific peripheral blood lymphocyte and myeloid cell subpopulations to the aging and pathology of heart and vessels. In a comprehensive report, TL was measured by flow-FISH in 12 leukocyte subsets obtained from age-matched healthy individuals and patients with coronary heart disease (CHD) (Spyridopoulos et al. 2008, 2009; Hoffmann et al. 2009). In both groups, TL in granulocytes and monocytes was comparable to that of their cell of origin, CD34-positive progenitor cells. LTL in CD34-positive progenitors, granulocytes, monocytes, and B and T lymphocytes was approximately 0.5 kb shorter in patients with CHD than in

controls. However, a twofold higher degree of telomere erosion was detected in cytotoxic CD8-positive T lymphocytes of CHD patients (Spyridopoulos et al. 2009). Moreover, TL shortening of this T lymphocyte subset in CHD patients was coupled with a decrease in left ventricular function.

In the majority of studies, patients with early-onset CHD and patients with severe complicated diabetes mellitus have shorter LTL than healthy subjects. A recent meta-analysis of 24 published reports was conducted to determine whether LTL is significantly associated with CHD and cerebrovascular disease (Haycock et al. 2014). This analysis involved 43,725 participants and 8,400 patients with cardiovascular disease. An inverse association between LTL and risk of CHD, independently from conventional vascular risk factors, was found. In contrast, shorter telomeres were not significantly associated with cerebrovascular disease risk. Moreover, the Halcyon study was designed to establish whether women have longer telomeres than men (Gardner et al. 2014). In 36 cohorts for a total of 36,230 participants, LTL was found to be longer in women, and this difference did not vary with chronological age. Conflicting data were obtained in patients with hypertension, in which elongated and shortened telomeres were identified; TL was preserved in patients with left ventricular hypertrophy (Nilsson et al. 2013).

Peripheral blood LTL has been considered as a systemic marker for biological aging, but the importance of telomere shortening as independent biomarker of organ and organism senescence has been challenged. Most studies agree that LTL adds predictive power to chronological age and can be considered a marker of cardiovascular aging (Fyhrquist et al. 2013).

The Telomerase-Telomere System in CPCs

The fraction of CPCs with critically shortened telomeres is the major determinant of the growth reserve of the heart (Leri et al. 2015). This is because hCPCs with shortened telomeres give rise to progenitors, precursors, and amplifying cells which inherit the characteristics of the mother cell and generate myocytes that rapidly acquire the senescent cell phenotype and express $p16^{INK4A}$. The link between the past history of CPCs, their telomere length, and the age of the formed progeny has been documented in a rigorous manner not only in humans (Cesselli et al. 2011) but also in small animal models of physiological aging (Sanada et al. 2014), strengthening the notion that myocardial biology and function are determined by the state of the controlling cell, i.e., the CPC. Aging supervenes when pro-senescence stimuli negatively affect cellular longevity, contributing to the physiological decline of the organ and organism.

Telomerase activity delays but cannot prevent telomere erosion in hCPCs, which lose ~130 bp at each round of division (Bearzi et al. 2007). With serial passaging, telomerase undergoes a 50 % decrease, but the catalytic activity remains at a considerably high level. A comprehensive evaluation of the biomarkers of cellular senescence was conducted in hCPCs isolated from the atrial myocardium of donor and explanted hearts (Cesselli et al. 2011). hCPCs obtained from explanted tissue

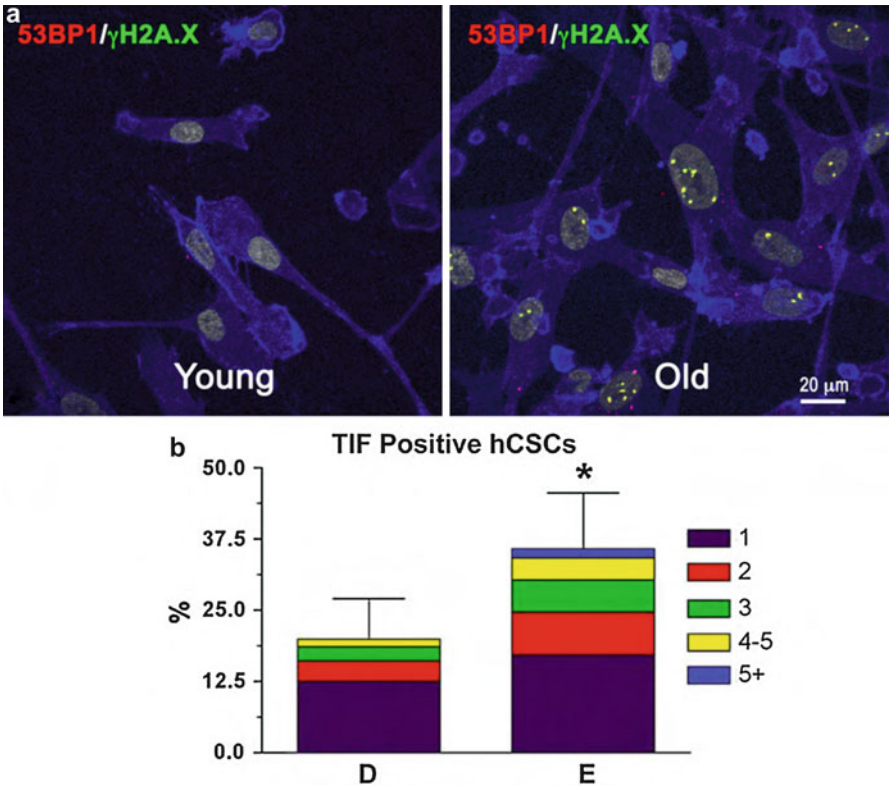


Fig. 10 DNA damage response (DDR) foci in c-kit-CPCs. (**a**) DDR foci labeled by γH2A.X (*green*) and 53BP1 (*red*) are more frequent in old CPCs. (**b**) The co-localization of telomere and DDR proteins was measured in hCSCs. The fraction of hCSCs with one to five TIFs is shown as mean ± SD. $*P < 0.05$ versus donor hearts (*D*). E: Explanted hearts (Panel **b** from Cesselli et al. 2011)

display a fivefold lower level of telomerase activity and a 25 % shorter TL. Although telomerase activity and average telomere length are valid indicators of the age of a cell compartment, the fraction of cells with critically shortened telomeres is the major determinant of the growth reserve of a cell population. Telomere erosion beyond a critical value and/or loss of telomere integrity elicits a DNA damage response that enables cells to block cell cycle progression and initiate DNA repair. The DNA damage response involves interaction of the adaptor protein p53-binding protein 1 (53BP1) and the chromatin modifier phosphorylated histone H2AX (γ-H2AX). The localization of these proteins within telomeric sequences reflects dysfunctional telomere-induced foci (TIFs) (Fig. 10a). TIFs activate the ataxia telangiectasia mutated (ATM) kinase which phosphorylates p53 at serine 15.

With respect to hCPCs from donor hearts, a 75 % larger fraction of hCPCs from explanted hearts showed TIFs in their telomeres (Fig. 10b). Consistent with these

observations, the quantity of phospho-p53^{Ser15} and the expression of the p53 target gene p21^{Cip1} were nearly twofold higher in explanted hCPCs. Importantly, a twofold larger fraction of hCPCs from explanted hearts was positive for the senescence-associated protein p16^{INK4a}. Thus, prolonged pathology and aging have comparable effects on hCPCs that display dysfunctional telomeres and express markers of cellular senescence.

Based on the assumption that telomere length, telomerase activity, TIFs, p16^{INK4a}, and p21^{Cip1} are biomarkers of hCPC function, their interrelation was established. Telomere length was directly related to telomerase activity and inversely correlated with TIFs, p16^{INK4a}, and p21^{Cip1}. Additionally, the level of catalytic activity of telomerase decreased with increased number of hCPCs showing TIFs, p16^{INK4a}, and p21^{Cip1}. Positive relationships were also found among TIFs, p16^{INK4a}, and p21^{Cip1}, indicating that these parameters of stem cell behavior were not independent; each of them could be used as a biomarker of the growth reserve of hCPCs.

Chronological age was recognized as a major predictor of telomere shortening, attenuation in telomerase activity, and increased incidence of TIFs, p16^{INK4a}, and p21^{Cip1} in hCPCs. To establish whether the presence of cardiac disease negatively affected hCPC function, hCPCs from subjects of comparable age were studied. In comparison with hCPCs from donor hearts, hCPCs from age-matched explanted hearts had shorter telomere length, lower telomerase activity, higher frequency of TIFs, and enhanced expression of p16^{INK4a} and p21^{Cip1}. These results suggest that both aging and pathological insults trigger DNA lesions at the level of the telomeric repeats with initiation of the DNA repair response. Activation of telomerase was apparent in hCPCs from control hearts, and this molecular mechanism may preserve partly telomere integrity.

The young heart is characterized by asymmetric growth kinetics of CPCs, a process that has been defined “invariant” and is operative in organs in a steady state. Changes in this pattern of growth have been observed in the old and diseased heart, suggesting that quantitative and qualitative alterations occur in hCPCs. Thus, the human heart is a self-renewing organ regulated by a CSC pool; CSCs condition the destiny of the organ throughout its life span and in the presence of various cardiac pathologies.

Potential Applications to Prognosis, Other Diseases, or Conditions

The discovery that the adult heart contains a compartment of resident CSCs has changed our understanding of myocardial biology and has projected a rather unexpected view of the growth reserve mechanisms of the myocardium. The discussion above has analyzed the variables that can affect the turnover of cardiac cells physiologically and the possibility of cardiac repair following myocardial damage. More importantly, the critical determinants of CSC replication, senescence, and death can be prospected as novel biomarkers able to define the age, pathology, and function of the myocardium with aging and heart failure. The molecular signature

and the telomere-telomerase system are proposed as relevant biomarkers describing the fate of the organ.

Summary Points

- This chapter focuses on the identifiers of the phenotypic and functional properties of cardiac progenitor cells (CPCs).
- CPCs are a rare population of cells that reside in the myocardium where they are clustered in niches.
- Stem cells are self-renewing, clonogenic, and multipotent, which are the fundamental properties of tissue-specific adult stem cell.
- CPCs regulate myocardial homeostasis and tissue repair following injury by generating cardiomyocytes and coronary resistance arterioles and coronary capillaries.
- c-kit-CPCs constitute a pool of undifferentiated cells while SP-CPCs and Sca1-CPCs show markers of early myocyte commitment.
- Transcriptional profile of c-kit-CPCs differs in normal and failing human hearts.

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Virtual Histology (VH) for Detecting Necrotic Core (NC)

37

Giancarla Scalone, Salvatore Brugaletta, and Manel Sabaté

Contents

Abstract

The ultimate characteristics of an atherosclerosis plaque at any given time depend on the relative contribution of its components. In particular, necrolipidic core is known to be the most thrombogenic component of atheromatous plaque. Therefore, the presence of fibroatheroma with a lipid-rich necrotic core and a thin fibrous cap (TCFA) appears particularly prone to rupture and subsequent coronary artery occlusion. Coronary angiography is unable to assess the magnitude

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_30

and composition of atherosclerotic burden. The recent development of novel intracoronary imaging methods able to detect plaque composition, such as intravascular ultrasound virtual histology (VH-IVUS), may represent a breakthrough in acute coronary syndrome prevention. In the future, a more extensive use of multiple image technologies in a single catheter, as VHS-IVUS and optical coherence tomography or near-infrared spectroscopy, will be likely to provide a more comprehensive assessment of the coronary vasculature. These imaging technologies and their clinical/research applications are discussed in detail in this section.

Keywords
Intravascular ultrasound imaging • Virtual histology • Necrotic core

Abbreviations

ACS	Acute coronary syndrome
BVS	Bioabsorbable stent
DES	Drug eluting stent
HR	Hazard ratio
IVUS-VH	Intravascular ultrasound virtual histology
LCBI	Lipid-core burden index
LCP	Lipid core plaque
LDL	Low-density lipoprotein/cholesterol
MACE	Major acute coronary events
MI	Myocardial infarction
NCCL	Necrotic core in contact with the lumen
NIRS	Near-infrared spectroscopy
OCT	Optical coherence tomography
PAV	Percent atheroma volume
PCI	Percutaneous coronary intervention
RF	Radiofrequency
ROC	Receiver-operating characteristic curve
SA	Stable angina
TCFA	Thin cap fibroatheroma

Key Factors of Vulnerable Plaque

- Acute coronary syndromes are mostly caused by sudden coronary thrombosis due to rupture of vulnerable plaques with a large lipid core or by erosion of endothelium within fibrous plaques.
- The precursor lesion of symptomatic heart disease is characterized by a large necrotic core and an overlying fibrous cap thinner than <65 μm, and is rich in inflammatory cells with a few smooth muscle cells.
- The necrolipidic core is known as the most thrombogenic component of atheromatous plaque due to its high content in tissue factor.

- Optical coherence tomography can provide a high-resolution image (10–20 μm) of the thin fibrous cap, but it is not able to detected a large and deep lipid core because of its limited penetration (<2 mm).

Key Factors of Necrotic Core

- Necrotic core of the coronary plaque has been variously related to clinical risk factors, and also associated with cardiac adverse events.
- The detection of plaques with high risk of rupture could prevent future occurrence of acute coronary syndrome.
- Virtual histology actually represents the gold standard to study the presence of necrotic core in the atherosclerotic plaque.

Definitions

Atheroma Atheromatous plaques are formed by an intricate sequence of events, not necessarily in a linear chronological order, that involves extracellular lipid accumulation, endothelial dysfunction, leukocyte recruitment, intracellular lipid accumulation (foam cells), smooth muscle cell migration and proliferation, expansion of extracellular matrix, neo-angiogenesis, tissue necrosis, and mineralization at later stages. The ultimate characteristics of an atherosclerosis plaque at any given time depend on the relative contribution of each of these features.

Coronary angiography Coronary angiography is a procedure that uses X-ray imaging and depicts arteries as a planar silhouette of the contrast-filled lumen. Angiographic disease assessment is based on the comparison of the stenotic segment with the adjacent, "normal appearing" coronary. It does not provide visualization of the vessel wall and is not suitable for assessment of atherosclerosis. Moreover, angiography interpretation is flawed by large inter- and intra-observer variability and usually underestimates the severity of the disease and vessel dimensions.

Intravascular ultrasound (IVUS) It is a three-dimensional imaging modality which provides a complete assessment of the coronary vessel wall. The IVUS image is the result of reflected ultrasound waves that are converted to electrical signals and sent to an external processing system for amplification, filtering, and scan-conversion. Its axial resolution is approximately 100 μm, while lateral resolution reaches 200–250 μm in conventional IVUS system (20–40 MHz).

IVUS-based imaging modalities IVUS-based imaging modalities are virtual histology, iMAP, integrated backscattered IVUS, and echogenicity. IVUS gray scale uses only the amplitude of the signal, while most of the IVUS-based imaging modalities use the radiofrequency data that lies underneath the amplitude.

Lipid core plaque (LCP) Lipid core plaque of interest is defined as a lipid core >60° in circumferential extent, >200-μm thick, with a mean fibrous cap thickness <450 μm.

Near-infrared spectroscopy catheter system (NIRS) NIRS is an imaging modality that uses near-infrared light to detect lipid. Since NIRS is coupled with an IVUS probe, the geometry of the plaques can be assessed simultaneously. It does not provide information about inflammation nor about other tissue types apart from lipid. Indeed, this NIRS catheter allows to analyze lipid core plaque "in vivo" because it can penetrate the blood and several millimeters into the tissue.

Necrotic core From the histological point of view, necrotic core can be defined as an area in which the extracellular matrix is lacking (total loss of collagen by picrosirius red staining) and replaced by dead cells and cellular debris (fragmented nuclei by hematoxylin and eosin staining). It is known to be the most thrombogenic component of atheromatous plaques, largely due to its high content in tissue factor, which sets off the exogenous coagulation cascade and is a major determinant in the equilibrium between pro- and anticoagulant processes.

Optical coherence tomography (OCT) OCT is a light-based imaging modality that can be applied in vivo in coronary arteries. By using reflected light instead of sound, OCT is able to provide images with a level of resolution (range 10–40 μm) tenfold higher than conventional IVUS. OCT can offer very valuable structural and compositional information about plaques causing coronary stenosis. It can accurately identify features related with culprit plaques in acute coronary syndromes such as plaque rupture and subsequent thrombosis. Furthermore, its ability to measure the fibrous cap and to detect macrophages makes of OCT one of the most promising techniques for the detection of plaques at high risk of rupture.

Thin fibrous cap atheroma (TCFA) A thin, fibrous cap (<65 μm) infiltrated by macrophages and lymphocytes with rare or absence of smooth muscle cells and a relatively large underlying necrotic core; intraplaque hemorrhage/fibrin may be present.

Virtual histology (VH-IVUS) Virtual histology is a spectral analysis of radiofrequency ultrasound signals using radiofrequency data. It provides information on four tissue types: fibrous, fibrofatty, necrotic core, and dense calcium. Evaluation of the layout of these four different tissue types gives information on different coronary plaque types. As virtual histology is an IVUS-derived technique, its axial resolution is limited (its axial resolution is approximately 100 μm, while lateral resolution reaches 250 μm). This precludes assessment of thin fibrous cap. Moreover, virtual histology does not provide information on thrombus and inflammation.

Introduction

The In Vivo Assessment of Atheroma Plaque

Atherosclerotic process is characterized by an intricate sequence of events that involve: extracellular lipid accumulation, endothelial dysfunction, leukocyte recruitment, intracellular lipid accumulation (foam cells), smooth muscle cell migration and proliferation, expansion of the extracellular matrix, neo-angiogenesis, tissue necrosis, and mineralization in the later stages. The ultimate characteristics of an atherosclerotic plaque at any given time depend on the relative contribution of each of these features (Virmani et al. 2000; Ross 1999). In this context, fibroatheroma with a lipid-rich necrotic core and a thin fibrous cap (TCFA) seems particularly prone to rupture and subsequently to coronary artery occlusion (Fernández-Ortiz et al. 1994). Indeed, the necrolipidic core is known as the most thrombogenic component of atheromatous plaques (Farb et al. 1996). This characteristic is mostly due to its high content in tissue factor (Thiruvikraman et al. 1996; Toschi et al. 1997), which sets off the exogenous coagulation cascade and represents a major determinant in the equilibrium between pro- and anticoagulant processes (Banner et al. 1996). TCFA is characterized by a large necrotic core containing numerous cholesterol clefts, cellular debris, and microcalcifications. In particular, the overlying fibrous cap is thin and rich in inflammatory cells, macrophages, and T lymphocytes with a few smooth muscle cells. Paralleling at the discovering of such a heterogeneous nature of the atheroma, new strategies to evaluate plaque composition and vessel architecture are emerging. Of note, coronary angiography depicts arteries as a planar silhouette of the contrast-filled lumen, therefore does not allow to assess atherosclerosis and to visualize vessel wall. Moreover, angiography interpretation is flawed by large inter- and intra-observer variability and usually underestimates the severity of the vessel disease, as well as its dimensions. Although quantitative coronary angiography has reduced the visual error, positive remodeling phenomenon makes angiography an unreliable method to assess atherosclerosis burden (Roberts and Jones 1979; Escaned et al. 1996).

In this context, grayscale intravascular ultrasound imaging (IVUS) overcomes the limitations of angiography and it is currently considered the gold standard for in vivo imaging of the wall of coronary arteries (Fujii et al. 2013; Mintz et al. 2001). In addition, it can be useful in various clinical scenarios: assessment of vessel remodeling, plaque progression/regression, ambiguous disease in vessels with aneurysmal dilatation, ostial stenoses, disease located at branching points or in the left main, tortuous or calcified segments, eccentric disease, complex disease morphology, intraluminal filling defects, thrombus, dissection, and lumen dimensions after coronary intervention (Sabaté et al. 1999; Jiménez-Quevedo et al. 2006; Futamatsu et al. 2006; Jiménez-Quevedo et al. 2005; Kay et al. 2000b).

The IVUS image is the result of reflected ultrasound waves that are converted to electrical signals and sent to an external processing system for amplification,

filtering, and scan-conversion. After placing the transducer, the beam remains almost parallel for a short distance ("near field"; better image quality) and then begins to diverge ("far field"). After encountering a transition between different materials, the beam will be partially reflected and partially transmitted, depending on tissue composition and differences in mechanical impedance between materials. Ultimately, grayscale IVUS imaging is formed by the envelope (amplitude) of the obtained radiofrequency signal.

The quality of ultrasound images is described by spatial and contrast resolution. In particular, its axial and lateral resolution reach approximately 100 and 200–250 μm in conventional IVUS system (20–40 MHz), respectively. Contrast resolution is the distribution of the gray scale of the reflected signal, and is often referred to as dynamic range.

An image of low dynamic range appears as black and white with a few levels of gray; images at high dynamic range are often softer.

However, the grayscale representation of the coronary vessel wall and plaque morphology, in combination with the limited resolution of the current IVUS catheter, makes difficult, if not impossible, to qualitatively identify the plaque morphology similarly to histopathology (Garcia-Garcia et al. 2010). For these reasons, virtual histology IVUS (VH-IVUS), an IVUS-based tissue characterization technique, has been designed to overcome these limitations. Indeed, it is able to identify the necrotic core of the coronary plaque, recently shown related to clinical risk factors and also associated with future cardiac adverse events (Nair et al. 2002, 2007; Garcia-Garcia et al. 2009a).

Tissue Characterization Using VH-IVUS

The first commercially available radiofrequency (RF) signal based tissue composition analysis tool was the so-called VH-IVUS (Volcano Therapeutics) software. It uses in-depth analysis of the backscattered RF signal in order to provide a more detailed description of the atheromatous plaque's composition. It is performed with either a 20 MHz, 2.9 F phased-array transducer catheter (Eagle EyeTM Gold, Volcano Therapeutics) or 45 MHz 3.2 F rotational catheter (Revolution, Volcano Therapeutics) that acquires ECG-gated IVUS data (Garcia-Garcia et al. 2009a). The main principle of this technique is to employ envelope amplitude of the reflected RF signals (as grayscale IVUS does) as well as underlie frequency content to analyze the tissue components present in the coronary plaque (Fig. 1). This combined information is processed using autoregressive models and thereafter in a classification tree that determines four basic plaque tissue components (Nair et al. 2007): (1) fibrous tissue (dark green), (2) fibrofatty tissue (light green), (3) necrotic core (red), and (4) dense calcium (white). The current software version assumes the presence of a media layer, artificially added and positioned just inside the outer vessel contour. This technique has been compared in several studies against histology in humans and other species (Table 1) (Nair et al. 2007; Brugaletta and Sabate 2014; Nasu et al. 2006; Granada et al. 2007; Van Herck et al. 2009; Thim et al. 2010) In

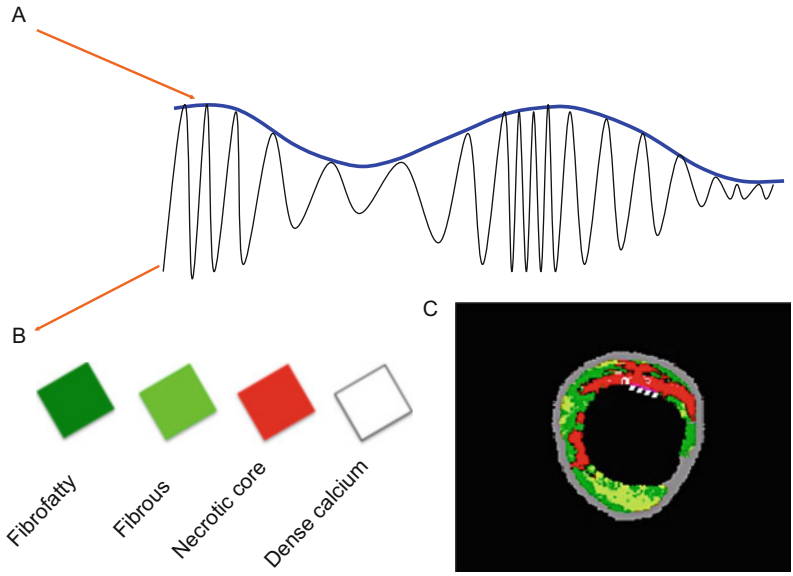

Fig. 1 Grayscale IVUS imaging is formed by the envelope (amplitude) (*A*) of the radiofrequency signal (*B*). The frequency and power of the signal commonly differ between tissues, regardless of similarities in amplitude. From the backscatter radiofrequency, virtual histology is obtained (*C*) and is able to detect four tissue types: fibrofatty, fibrous, necrotic core, and dense calcium

particular, VH-IVUS spectral analysis correlates well with histopathology, with predictive accuracy of 87.1 %, 87.1 %, 88.3 %, and 96.5 % for fibrous, fibrofatty, necrotic core, and dense calcium, respectively (Brugaletta et al. 2014; Nasu et al. 2006; Granada et al. 2007; Van Herck et al. 2009; Thim et al. 2010).

VH-IVUS Diagnostic Application

Plaque Characterization

VH-IVUS (Fig. 2) is able to define the various phases of atherosclerosis (Garcia-Garcia et al. 2006). More specifically, the definition of an "IVUS-derived TCFA" consists in a lesion fulfilling the following criteria in at least three consecutive frames: (1) plaque burden >40 %, and (2) confluent necrotic core >10 % in direct contact with the lumen (i.e., no visible overlying tissue) (Garcia-Garcia et al. 2006). Using this refined definition of "IVUS derived TCFA," in patients with acute coronary syndrome (ACS) who underwent IVUS of all three epicardial coronaries, there were "2 IVUS-derived TCFA" per patient, with half of them showing outward remodeling. Accordingly, Hong et al. (2008) described the frequency and distribution of TCFA in a 3-vessel VH-IVUS study of patients with ACS ($n = 105$) or stable angina (SA; $n = 107$). There were 2.5 ± 1.5 TCFAs per patient in the ACS group

Table 1 Comparison of VH-IVUS with histology in humans and other species

Author	Type of study	Year	Principal results
Nair et al.	Ex vivo	2002	Coronary plaque classification with IVUS RF data analysis: Autoregressive classification schemes performed better than those from classic Fourier spectra with accuracies of 90.4 % for FT, 92.8 % for fibrolipidic, 90.9 % for calcified, and 89.5 % for calcified-necrotic regions in the training data set and 79.7 %, 81.2 %, 92.8 %, and 85.5 %, respectively, in the test data
Nasu et al.	In vivo	2006	Accuracy of in vivo coronary plaque morphology assessment: a validation study of in vivo VH compared with in vitro histopathology. Predictive accuracy from all patients data: 87.1 % for FT, 87.1 % for FF, 88.3 % for NC, and 96.5 % for DC regions, respectively. Sensitivities: NC 67.3 %, FT 86 %, FF 79.3 %, DC 50 %. Specificities: NC 92.9 %, FT 90.5 %, FF 100 %, DC 99 %
Nair et al.	Ex vivo	2007	Automated coronary plaque characterization with IVUS backscatter: ex vivo validation. Overall predictive accuracies were 93.5 % for FT, 94.1 % for FF, 95.8 % for NC, and 96.7 % for DC. Sensitivities: NC 91.7 %, FT 95.7 %, FF 72.3 %, DC 86.5 %. Specificities: NC 96.6 %, FT 90.9 %, FF 97.9 %, DC 98.9 %
Granada et al.	In vivo	2007	In vivo plaque characterization using VH-IVUS in a porcine model of complex coronary lesions: compared with histology, VH-IVUS correctly identified the presence of FT, FF, and necrotic tissue in 58.33 %, 38.33 %, and 38.33 % of lesions, respectively. Sensitivities: FT 76.1 %, FF 46 %, and NC 41.1 %
Van Herk et al.	In vivo	2009	Validation of in vivo plaque characterization by VH in a rabbit model of atherosclerosis: VH-IVUS had a high sensitivity, specificity, and positive predictive value for the detection of noncalcified TCFA (88 %, 96 %, 87 %, respectively) and calcified TCFA (95 %, 99 %, 93 %, respectively). These values were respectively 82 %, 94 %, 85 % for noncalcified FA and 78 %, 98 %, 84 % for calcified FA. The lowest values were obtained for pathologic intimal thickening (74 %, 92 %, 70 %, respectively). For all plaque types, VH-IVUS had a kappa-value of 0.79
Thim et al.	Ex vivo	2010	Unreliable assessment of NC by VHTM IVUS in porcine coronary artery disease: no correlations were found between the size of the NC determined by VH-IVUS and histology. VH-IVUS displayed NCs in lesions lacking cores by histology
Brugaletta et al.	In vivo	2014	Qualitative and quantitative accuracy of VH-IVUS for detection of NC in human coronary arteries: VH had high sensitivity, but low specificity and low positive predictive value for NC identified by histology. In addition, it was not able to accurately quantify its size in the histological specimen

DC dense calcium, *FA* fibroatheroma, *FF* fibrofatty, *FT* fibrous tissue, *IVUS* intravascular ultrasound, *NC* necrotic core, *RF* radiofrequency, *TFCA* thin fibrous cap atheroma, *VH* virtual histology

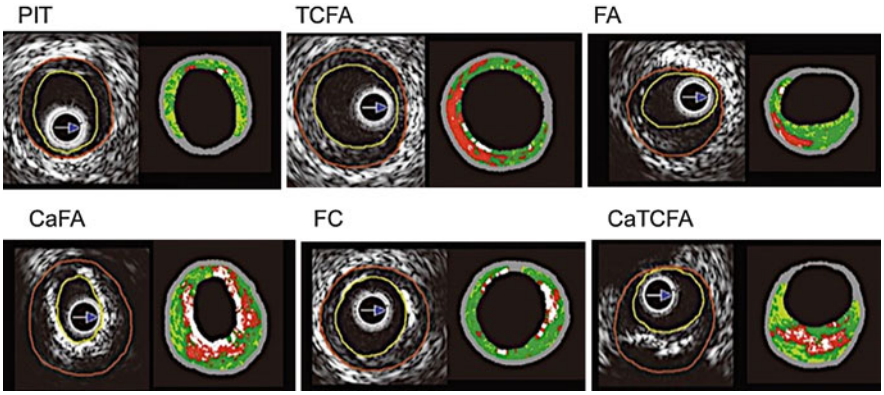

Fig. 2 Examples of various atherosclerotic plaques in different stages, classified by virtual histology (VH). Lumen contour (*yellow line*) and vessel contour (*red line*) are shown. In the VH images, necrotic core is coded as *red*, dense calcium as *white*, fibrous tissue as *dark green*, and fibrofatty tissue as *light green. CaFA* calcified fibroatheroma, *CaTCFA* calcified thin-cap fibroatheroma, *FA* fibroatheroma, *FC* fibrocalcific plaque, *PIT* pathological intimal thickening, *TCFA* thin-cap fibroatheroma

and 1.7 ± 1.1 in the SA group, respectively ($P < 0.001$). Presentation of ACS was the only independent predictor for multiple VH-derived TCFA (VH-TCFA) ($P = 0.011$), and 83 % of VH-TCFAs were located within the first 40 mm of the coronary artery. By use of VH-IVUS, the serial changes in VH plaque type have been also investigated. In particular, Kubo et al. (2010) showed that most VH-TCFAs healed during a 12-month follow-up. However, along the follow-up period, new VH-TCFA developed and pathologic intimal thickening and necrotic core plaques had a significant progression compared with fibrotic and fibrocalcific plaques in terms of increase in plaque area and decrease in lumen. Moreover, a recent study using VH demonstrated that, in patients with ST elevation myocardial infarction after thrombolysis, the necrotic core content of culprit plaques is strongly associated with the degree of flow restoration. Indeed, there were significant differences in the relative necrotic core content, both in proportion to the whole plaque volume (26.3 % vs. 29.9 %; $p = 0.016$), as well as in area fraction at the largest necrotic core site (31.5 % vs. 40.3 %; $p < 0.001$) between patients with TIMI 3 versus those with TIMI 1–2 flow grade (Giannopoulos et al. 2014).

The potential value of VH-TCFA in the prediction of adverse coronary events was evaluated in an international multicenter prospective study, the Providing Regional Observations to Study Predictors of Events in the Coronary Tree study (PROSPECT study) (Stone et al. 2011).

The PROSPECT trial was conducted in ACS patients, all of whom underwent percutaneous coronary intervention (PCI) for their culprit lesion at baseline followed by angiography and VH-IVUS of the three major coronary arteries. A TCFA with a minimum luminal area ≤ 4 μm^2 and a large plaque burden ≥ 70 % had a 17.2 % likelihood of causing an event within 3 years. Interestingly, the anticipated high

frequency of acute thrombotic cardiovascular events did not occur, with only a 1 % rate of myocardial infarction (MI) and no deaths directly attributable to nonculprit vessels over the 3 years of follow-up. These results suggest that nonculprit yet obstructive coronary plaques were most likely to be associated with increasing symptoms rather than thrombotic acute events, with 8.5 % of patients presenting with worsening angina and 3.3 % with unstable angina. Of note, the findings of the PROSPECT trial were not translated in clinical practice into a percutaneous preventive treatment of VH-TCFA.

These results were recently confirmed by the VIVA study (Calvert et al. 2011). In particular, this study aimed at determining whether TCFA identified by VH-IVUS are associated with major adverse cardiac events (MACE) on individual plaque or whole patient analysis. For this purpose, 1070 with SA or troponin-positive ACS referred for PCI were prospectively enrolled and underwent 3-vessel VH-IVUS pre-PCI and also post-PCI in the culprit vessel. MACE consisted of death, MI, or unplanned revascularization. In all, 30,372 mm of VH-IVUS were analyzed. Eighteen MACE occurred in 16 patients over a median follow-up of 625 days (interquartile range, IR: 463–990 days); 1,096 plaques were classified, and 19 lesions resulted in MACE (13 nonculprit lesions and 6 culprit lesions). Nonculprit lesion factors associated with nonrestenotic MACE included VH-TCFA (hazard ratio [HR]: 7.53, $p = 0.038$) and plaque burden >70 % (HR: 8.13, $p = 0.011$). VH-TCFA (HR: 8.16, $p = 0.007$), plaque burden >70 % (HR: 7.48, $p = 0.001$), and minimum luminal area <4 μm^2 (HR: 2.91, $p = 0.036$) were associated with total MACE. On patient-based analysis, the only factor associated with nonrestenotic MACE was 3-vessel noncalcified VH-TCFA (HR: 1.79, $p = 0.004$). The crucial issue of this study was represented by the association between VH-IVUS–based plaque classification and total and nonrestenotic MACE. In particular, nonculprit lesion plaque burden >70 % and remodeling index showed a strong correlation with nonrestenotic MACE. These results emphasize the biological importance of this association, and indicate that VH-IVUS can identify plaques at increased risk of subsequent events (Calvert et al. 2011).

VH-IVUS Research Application

Assessment of Drug Effect on Atherosclerosis Progression/Regression

VH-IVUS has so far been used in various studies to show serial changes of plaque composition in patients treated with various drugs (Table 2). In particular, Nasu et al. (2009) demonstrated that treatment with fluvastatin for 1 year in patients with SA ($n = 80$) caused a significant regression of the plaque volume and caused changes in the atherosclerotic plaque composition with a significant reduction of the fibrofatty volume ($P < 0.0001$). This change in fibrofatty volume had a significant correlation with changes in the low-density lipoprotein/cholesterol (LDL) level ($r = 0.703$, $P < 0.0001$) and in the high-sensitivity C-reactive protein level ($r = 0.357$, $P = 0.006$) (Nasu et al. 2009; Kovarnik et al. 2012). Of note, the

Table 2 Serial changes of plaque composition by VH-IVUS in patients treated with various statins

Author	Type	Year	Treatment	Patients	Period	Parameter	Results
Serruyus et al.	RCT	2008	Darapladib placebo	175 155	12 months	NC volume by VH-IVUS	Darapladib reduced NC
Nasu et al.	Obs	2009	Fluvastatin Control	40 40	12 months	Overall tissue characterization by VH-IVUS	Fluvastatin reduced plaque and FF volume
Hong et al.	RCT	2009	Simvastatin Rosuvastatin	50 50	12 months	Overall tissue characterization by VH-IVUS	Both reduced NC and increased FF volume
Toi et al.	RCT	2009	Atorvastatin Pitavastatin	80 80	2–3 week	Overall tissue characterization by VH-IVUS	Pitavastatin reduced plaque and FF volumes
Räber et al. (2015)	Obs	2014	Rosuvastatin	103	13 months	Overall tissue characterization by IVUS and RF-IVUS	Rosuvastatin reduced coronary atherosclerosis without change in RF-IVUS defined NC or plaque phenotype

FF fibrofatty, *NC* necrotic core, *OBS* observational, *RCT* randomized controlled trial, *RF* radiofrequency, *VH-IVUS* virtual histology intravascular ultrasound

necrotic core did not change significantly. The same data were found with the use of pitavastatin (Toi et al. 2009). In another study, Hong et al. (2009) randomized 100 patients with SA and ACS to either rosuvastatin 10 mg or simvastatin 20 mg for 1 year, showing a significant decrease of overall necrotic core volume ($P = 0.010$) and an increase of fibrofatty plaque volume ($P = 0.006$) after statin treatment. In particular, there was a decrease in the necrotic core volume ($P = 0.015$) in the rosuvastatin-treated subgroup. Results from multiple stepwise logistic regression analysis identified the baseline high-density lipoprotein/cholesterol level as the only independent clinical predictor of decrease in the necrotic core volume ($P = 0.040$, odds ratio 1.044, 95 % confidence interval 1.002–1.089).

Again, the IBIS-2 study (Serruys et al. 2008) compared the effects of 12 months of treatment with darapladib (oral Lp-PLA2 inhibitor, 160 mg daily) versus placebo in 330 stable and no-stable patients. In placebo group, necrotic core volume increased significantly, whereas darapladib halted this increase, resulting in a significant treatment difference of -5.2 mm^3 ($P = 0.012$). Remarkably, these intraplaque compositional changes occurred without a significant treatment difference in total atheroma volume. However, despite all these data, a direct association between a decrease in plaque size and/or plaque composition and a reduction in clinical events has not yet been described. Indeed, the latest studies revealed that darapladib did not decrease the risk of MACE both in patients with ACS (O'Donoghue et al. 2014) and with stable coronary artery disease (White et al. 2014). The best attempt using serial IVUS was a pooled analysis of 4,137 patients from six clinical trials (Nicholls et al. 2010); percent atheroma volume (PAV) increased by 0.3 % ($p < 0.001$) and 19.9 % of subjects experienced MACE (0.9 % death, 1.8 % MI, 18.9 % coronary revascularization). Greater baseline PAVs were observed in patients who experienced MI (42.2 +/− 9.6 % vs. 38.6 +/− 9.1 %, $p = 0.001$), coronary revascularization (41.2 +/− 9.3 % vs. 38.1 +/− 9.0 %, $p < 0.001$), or MACE (41.3 +/− 9.2 % vs. 38.0 +/− 9.0 %, $p < 0.001$). Each standard deviation increase in PAV was associated with a 1.32-fold (95 % confidence interval, CI: 1.22–1.42; $p < 0.001$) greater likelihood of experiencing a MACE. During follow-up (21.1 +/− 3.7 months), PAV but not total atheroma volume was greatly increased in subjects who experienced MACE compared with those who did not (0.95 +/− 0.19 % vs. 0.46 +/− 0.16 %, $p < 0.001$). Each standard deviation increase in PAV was associated with a 1.20-fold (95 % confidence interval: 1.10–1.31; $p < 0.001$) greater risk for MACE. Multivariate analysis revealed that factors associated with MACE included baseline PAV ($p < 0.0001$), change in PAV ($p = 0.002$), smoking ($p = 0.0002$), and hypertension ($p = 0.01$).

Recently, the IBIS4 aimed at quantifying the impact of high-intensity statin therapy (40 mg, day through 13 months) on plaque burden, composition, and phenotype in non–infarct-related arteries of 103 STEMI patients undergoing primary PCI, using IVUS and RF IVUS. After 13 months, low-density lipoprotein cholesterol decreased from a median of 3.29 to 1.89 mmol/L ($P < 0.001$), whereas high-density lipoprotein cholesterol levels increased from 1.10 to 1.20 mmol/L ($P < 0.001$). PAV of the non–infarct-related arteries decreased by -0.9 % (95 % CI: -1.56 to -0.25, $P = 0.007$). However, percent necrotic core remained

unchanged (−0.05 %, 95 % CI: −1.05 to 0.96 %, $P = 0.93$) as did the number of RF-IVUS defined TCFA (124 vs. 116, $P = 0.15$) (Räber et al. 2015).

Vascular Response to Endovascular Device

IVUS has been extensively used as surrogate endpoint in stent trials, primarily to assess effectiveness of devices as it relates to neo-intimal proliferation. IVUS was an essential investigational tool during initial clinical testing of drug eluting stent (DES) (Jiménez-Quevedo et al. 2006; Kay et al. 2000a), confirming the dramatic suppression of neo-intimal proliferation, revealing new patterns of restenosis and establishing intravascular imaging metrics of stent optimization.

Recently, the feasibility and safety of a bioabsorbable stent everolimus-eluting stent (BVS) was also assessed with intravascular imaging. In this context, a prospective open-label study, enrolling 30 patients with a single de novo lesion treated with BVS, showed IVUS-VH changes with reduction of RF backscattering by polymeric struts (Garcia-Garcia et al. 2009b) at 6 months follow-up. Again, another study by Brugaletta et al. (2011a) examined the temporal IVUS-VH changes in composition of the plaque behind the struts following BVS implantation, at 6 month follow-up. Compared to baseline, there was an increase in both the area of plaque behind the struts ($P = 0.005$) and the external elastic membrane area ($P = 0.006$). Furthermore, they showed a significant progression in the "necrotic core" ($P = 0.010$) and fibrous tissue area ($P = 0.027$). Hence, serial IVUS-VH analysis of BVS-treated lesions at 6 months follow-up demonstrated a progression in the necrotic core and fibrous tissue content of plaque behind the struts.

Combined Used of Multiple Image Technologies

VH-IVUS OCT Applications

In the future, integration of multiple image technologies in a single catheter is likely to provide a more comprehensive assessment of the coronary vasculature. In particular, the combined use of IVUS-VH analysis and optical coherence tomography (OCT) seems to improve the accuracy for TCFA assessment and the quantification of necrotic core (Gonzalo et al. 2009; Sawada et al. 2008). This represents a crucial point, considering that the detection of plaques with high risk of rupture could prevent future occurrence of ACS. On the one hand, if RF data analysis by VH-IVUS allows to classify the atherosclerotic plaques (fibrous, fibrofatty, dense calcium, and necrotic core) and quantify the necrotic core (Nair et al. 2007; Brugaletta et al. 2014), it cannot visualize the thin fibrous cap because of its limited resolution (>100 μm) (Sawada et al. 2008). Conversely OCT can provide a high-resolution image (10–20 μm) of the thin fibrous cap, but it is not able to detected a large and deep lipid core because of its limited penetration (<2 mm) (Matsumoto et al. 2007; Jang et al. 2002; Yabushita et al. 2002).

Sawada et al. (2008) delucidated the feasibility of the combined use of VH-IVUS and OCT to detect in vivo TCFA and to clarify the lesion characteristics of TCFA.

They identified, in 56 patients with angina, 126 plaques using both VH-IVUS and OCT. In particular, "IVUS-derived TCFA" was defined as an abundant necrotic core (>10 % of the cross-sectional area) in contact with the lumen (NCCL) and percentage plaque-volume >40 %, whereas "OCT-derived TCFA" was defined as a fibrous cap thickness of <65 μm overlying a low-intensity area with an unclear border. Plaque meeting both TCFA criteria was identified as "definite-TCFA." Sixty-one plaques were diagnosed as "IVUS-derived TCFA" and 36 plaques as "OCT-derived TCFA." Twenty-eight plaques were diagnosed as "definite-TCFA"; the remaining 33 "IVUS-derived TCFA" had a non-thin-cap and 8 "OCT-derived TCFA" had a non-NCCL. These lesions were characterized by a larger reference diameter, minimum lumen diameter, and minimum vessel volume ($p = 0.002$, $p = 0.004$, $p = 0.01$, respectively), as well as severely calcified components compared with "definite-TCFA" ($p = 0.01$). When a target vessel or plaque is large, the optical signal might be attenuated by the plaque and failed to identify plaque morphology. Furthermore, it might also be sometimes difficult to discriminate between lipid and calcified lesions because both these appear as low-intensity images, usually differentiated in OCT by an unclear border (lipid) or a clear border (calcium). In this regard, large calcified lesions are likely to be misdiagnosed as TCFA by OCT examination. Ultimately, the positive ratio of VH-IVUS for detecting definite-TCFA was 45.9 % and that for OCT 77.8 %. Hence, the use of complementary tools such as VH-IVUS and OCT might be a feasible approach for more accurate detection of TCFA.

NIRS-VH-IVUS Applications

A near-infrared spectroscopy (NIRS) catheter system (Lipiscan; InfraReDx Inc) has been recently developed for invasive detection of the lipid core in plaque (LCP) composition "in vivo" (Gardner et al. 2008; Waxman et al. 2009; Brugaletta et al. 2011b), penetrating the blood and several millimeters into the tissue. Moreover, it overcomes the problem of cardiac motion because it uses an ultrafast scanning laser and provides a chemical measure of the LCP target of interest (Garcia-Garcia et al. 2010; Moreno et al. 2002). In the last years, identification of LCP with NIRS has been showed to improve the safety of stenting, optimize the length of vessel to stent, ensure an adequate stent implantation, and also detect the lipid-core lesions at higher risk of distal embolization, possibly leading to effective use of distal embolic protection devices in the native coronaries (Oemrawsingh et al. 2003; Sakhuja et al. 2010; Waxman et al. 2010). Recently, a "head to head" comparison between VH-IVUS and NIRS for the identification of LCP/necrotic core rich plaques has been performed (Fig. 3). Larger coronary plaques, identified by grayscale IVUS, were more likely to be recognized as LCP and as necrotic-core rich plaques by NIRS and VH, respectively. However, the correlation between NIRS and VH was poor (Brugaletta et al. 2011b; Brugaletta and Sabaté 2014). Of note, for the validation of NIRS, LCP was defined as a fibroatheroma with lipid core >60° in circumferential extent, >200 μm thick, with a fibrous cap having a mean thickness <450 μm and correlated with each chemogram block (Gardner et al. 2008). On the contrary, for the

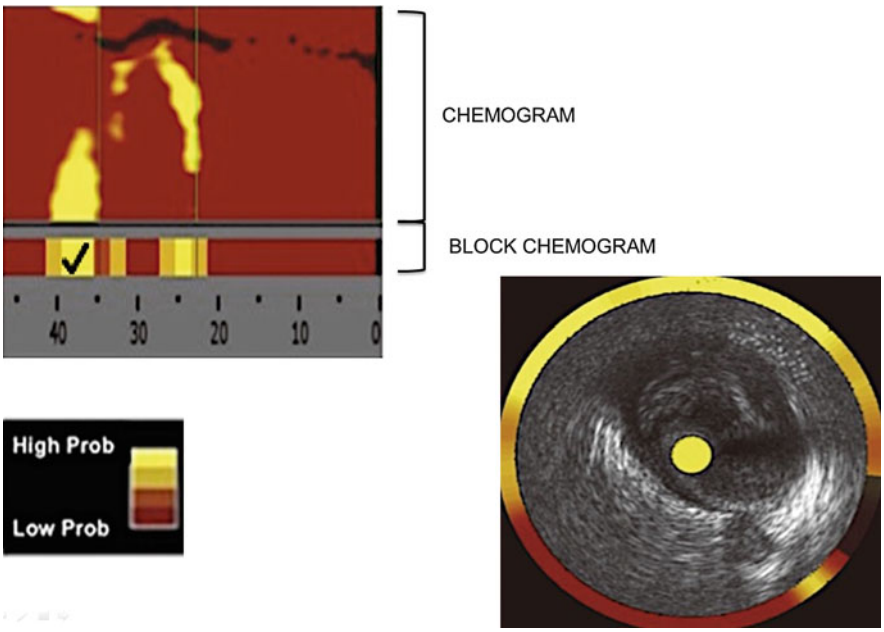

Fig. 3 At the *top left*, image obtained by NIRS displayed as a chemogram and block chemogram. The chemogram shows the scanned arterial segment as a map, whereas the block chemogram shows the presence of lipid core as a 2-mm segment using the top 90th percentile information within each 2-mm segment. The probability of lipid is displayed in a color scale from *red* (low probability) to *yellow* (high probability), through *orange* and *tan*. At the *bottom right*, acquisition with a combined NIRS-IVUS catheter. Surrounding the IVUS image, the colors define the presence of lipid (*red* = low probability/*yellow* = high probability). Note an IVUS plaque from 11 to 2 o'clock, coded as lipid-core rich by NIRS. *IVUS* intravascular ultrasound, *NIRS* near-infrared spectroscopy

validation of VH-IVUS, necrotic core was defined as the region comprising cholesterol clefts and foam cells. Some lipid components in the presence of collagen are also considered as fibrofatty tissue (Nair et al. 2002). Moreover, it should be taken in account that if VH is based on pattern classification of backscattering ultrasound signal, NIRS is based on near-infrared spectral signals. Ultimately, NIRS-IVUS device might be employed in the identification of vulnerable plaque (Brugaletta et al. 2012; Madder et al. 2013), and in the development of anti-atherosclerotic medications by providing a surrogate endpoint in plaque regression/stabilization studies (Simsek et al. 2012).

Conclusions

VH-IVUS has been shown to identify coronary plaque morphology similarly to histopathology. In particular, it is able to identify the necrotic core content that has been variously related to clinical risk factors, and also associated with the development of cardiac adverse events. In the next years, an extensive use of VH-IVUS,

alone or combined with other image technologies (e.g., OCT and NIRS), might improve the accuracy of TCFA assessment and the quantification of necrotic core.

Potential Applications to Prognosis, Other Diseases, or Conditions

The potential value of VH-TCFA in the prediction of adverse coronary events was evaluated in two recent studies: PROSPECT and VIVA trials.

In particular, the PROSPECT study showed that a TCFA with a minimum luminal area $\leq 4\ \mu m^2$ and a large plaque burden $\geq 70\ \%$ had a 17.2 % likelihood of causing an event within 3 years; the anticipated high frequency of acute thrombotic cardiovascular events did not occur, with only a 1 % rate MI and no deaths directly attributable to nonculprit vessels over the 3 years of follow-up. These results suggest that nonculprit, yet obstructive coronary plaques, were most likely to be associated with increasing symptoms rather than thrombotic acute events, with 8.5 % of patients presenting with worsening angina and 3.3 % with unstable angina.

More recently, the VIVA trial aimed at determining whether TCFA identified by VH-IVUS are associated with MACE on individual plaque or whole patient analysis. Out of 1,096 plaques classified, 19 lesions resulted in MACE (death, MI, or unplanned revascularization): 6 culprit and 13 nonculprit lesions. Nonculprit lesion factors associated with nonrestenotic MACE included VH-TCFA (HR: 7.53, $p = 0.038$) and plaque burden >70 % (HR: 8.13, $p = 0.011$). On the other hand, VH-TCFA (HR: 8.16, $p = 0.007$), plaque burden >70 % (HR: 7.48, $p = 0.001$), and minimum luminal area $<4\ \mu m^2$ (HR: 2.91, $p = 0.036$) were associated with total MACE. On patient-based analysis, the only factor associated with nonrestenotic MACE was 3-vessel noncalcified VH-TCFA (HR: 1.79, $p = 0.004$). In conclusion, this study showed an association between VH-IVUS–based plaque classification and total and nonrestenotic MACE. In particular, nonculprit lesion plaque burden >70 % and remodeling index showed a strong correlation with nonrestenotic MACE.

Summary Points

- This chapter is focused on virtual histology, an intravascular ultrasound based tissue characterization technique that provides a classification tree that determines four basic plaque tissue components: fibrous tissue (dark green), fibrofatty tissue (light green), necrotic core (red), and dense calcium (white).
- It is currently employed in the catheterization laboratory for diagnostic purpose as plaque characterization, whereas its research applications are represented by assessment of drug effect on atherosclerosis and response to endovascular devices.
- PROSPECT and VIVA trials showed that intravascular ultrasound virtual histology is able to identify the necrotic core of the coronary plaque that has been variously related to clinical risk factors and also to the risk of adverse events.
- The combined use of intravascular ultrasound virtual histology and other image technologies (e.g., optical coherence tomography and near-infrared spectroscopy)

will be likely to provide a more comprehensive assessment of the coronary vasculature.

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Biomarkers of Coronary Plaque Composition and Vulnerability

38

Leonardo De Luca and Fabrizio Tomai

Contents

Abstract

An important challenge to facing the epidemic of cardiovascular disease is the unpredictable nature of acute coronary events. Therefore, substantial research has been recently conducted in order to develop new methods to identify subjects at risk or atheromatous plaque that are prone to produce sudden major coronary events. Over the past two decades, the concept of "vulnerable plaque" has gained attention as a paradigm to improve risk stratification and potentially lead to the discovery of novel markers of risk to prevent cardiovascular disease.

We reviewed biochemical markers that have been investigated to date for the identification of coronary atherosclerotic plaque composition and early detection of their vulnerability. C-reactive protein and matrix metalloproteinases are the

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_44

most commonly studied, but also novel biomarkers reflecting a variety of pathophysiologic pathways such as ischemia, inflammation, vascular dysfunction, biomechanical stress, hemostasis, and lipid metabolism have been reported to be potentially associated with increased risk of coronary events.

Keywords

C-reactive protein • Coronary plaque • Matrix metalloproteinases • Percutaneous coronary intervention • Vulnerable plaque

Abbreviations

ACS	Acute coronary syndrome
CRP	C-reactive protein
CVD	Cardiovascular disease
LDL	Low-density lipoprotein
MACE	Major adverse cardiovascular events
MI	Myocardial infarction
MMP	Matrix metalloproteinases
PCI	Percutaneous coronary intervention
TCFA	Thin-cap fibroatheromas
VSMC	Vascular smooth muscle cells

Definitions

Acute coronary syndrome Any condition brought on by sudden, reduced blood flow to the heart

Culprit lesion The coronary lesion involved in the initial myocardial infarction

Nonculprit lesion Any lesion in the entire coronary tree outside the culprit lesion

Thin-cap fibroatheromas Lesions with a fibrous cap <65 μm with macrophage infiltration (>25 cells/high-magnification field) and an underlying necrotic core

Vulnerable plaque A kind of atheromatous plaque that is particularly unstable and prone to produce sudden major coronary events

Introduction

Atherosclerosis is a chronic condition with acute cardiovascular manifestations. For many patients, the first sign of atherosclerosis is an acute myocardial infarction (MI), sudden cardiac death, or a disabling stroke. An important challenge to facing the epidemic of cardiovascular disease is the unpredictable nature of its acute manifestations. Therefore, substantial research has been recently conducted in order to

develop new methods to identify subjects at risk before the occurrence of a cardiovascular event. Furthermore, among patients who have survived a cardiovascular event, the risk for a subsequent event remains relatively high, despite aggressive treatment (Cannon et al. 2004). Such recurrence rates highlight the need for novel approaches to secondary prevention of cardiovascular disease and to the treatment of index events.

Pathophysiology of Atherosclerotic Plaque

There has been considerable progress in the identification of the molecular and cellular processes causing atherosclerosis and its clinical sequelae (Daugherty et al. 2005; Libby et al. 2002). Low-density lipoprotein (LDL) cholesterol is central to the development of the disease. In addition, it is now clearly established that inflammation plays an important role in the initiation of lesions and is likely to be responsible for the activation of the disease in more than a single plaque or artery (Hansson et al. 2005; Libby 2005).

Chronic endothelial injury eventually results in endothelial dysfunction and increased permeability and induces LDL oxidation and accumulation in the subendothelial space of the intima as well as the expression of adhesion molecules and chemokines that participate in platelet aggregation and lymphocyte and monocyte adhesion and infiltration, thus initiating the inflammatory process (Daugherty et al. 2005; Libby et al. 2002; Hansson et al. 2005; Libby 2005). As monocytes are attracted to the endothelium and migrate to the subendothelial space, they mature into macrophages and uptake oxidized LDL transforming into "foam" cells that eventually form the lipid core of the atherosclerotic plaque after apoptosis occurs. This inflammatory mediator cascade promotes a phenotype change of vascular smooth muscle cells (VSMCs) from the "contractile" phenotype state to the active "synthetic" state. VSMCs in the synthetic state can migrate and proliferate from the media to the intima, where they produce excessive amounts of extracellular matrix (e.g., collagen, elastin, and proteoglycans) that transforms the lesion into a fibrous plaque (Daugherty et al. 2005; Libby et al. 2002; Hansson et al. 2005; Libby 2005). The typical atherosclerotic plaque comprises of the lipid core and the fibrous cap and is the most commonly classified histologically by the American Heart Association-recommended Stary classification (Stary 2000).

Vulnerable atherosclerotic plaques (high-risk or unstable plaques) are associated with an increased risk of disruption, distal embolization and vascular events. They are histological lesions with a large lipid core and a thin fibrous cap and may contain ulceration, intraluminal thrombosis, and intraplaque hemorrhage, as well as intense infiltration of macrophages and other inflammatory cells (Fig. 1).

Over the past two decades, the concept of "vulnerable plaque" has gained attention as a paradigm to improve risk stratification and potentially lead to newer invasive and noninvasive therapeutic options to prevent and treat cardiovascular disease (Alsheikh-Ali et al. 2010).

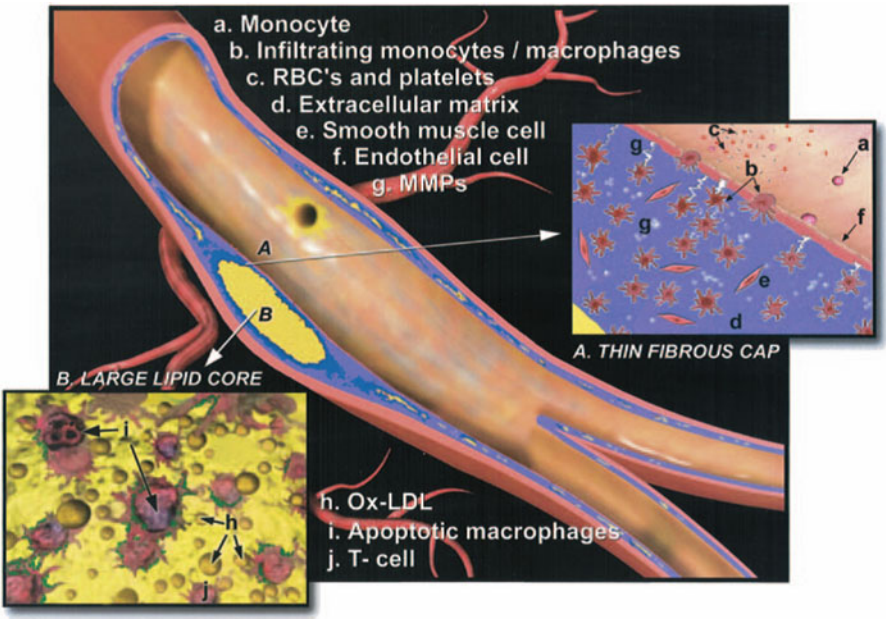


Fig. 1 Schematic figure illustrating the most common type of vulnerable plaque characterized by thin fibrous cap, extensive macrophage infiltration, paucity of smooth muscle cells, and large lipid core, without significant luminal narrowing (Reprinted with permission from Naghavi et al. 2003)

The Vulnerable Plaque

The term vulnerable plaque was first used more than two decades ago in the context of studying triggers of acute cardiovascular disease (Muller et al. 1989). Since its introduction, the term vulnerable plaque has been used interchangeably in reference to the concept of propensity to result in an acute cardiovascular event or to denote a plaque with the histologic hallmarks of culprit lesions from autopsy studies. A more broad definition was proposed in 2003 to include not only susceptibility to rupture but also susceptibility to thrombose or rapidly progress to a culprit lesion (Fig. 2), based on observations that rupture of plaques, although common in culprit lesions, is not universal (Naghavi et al. 2003). Indeed, almost one third of such lesions exhibit erosion or nodular calcification without rupture of the fibrous cap (Virmani et al. 2000). The introduction of this concept paralleled an increase in appreciation of the limitations of imaging arterial lumens and quantifying risk based merely on the severity of arterial stenoses. In several prospective and retrospective serial angiographic studies, the culprit lesion in nearly two thirds of patients with acute coronary events was shown to have less than 70 % (often $<$50 %) diameter narrowing on coronary angiography weeks or months before the index event (Ambrose et al. 1986; Little et al. 1988; Hackett et al. 1988; Giroud et al. 1992).

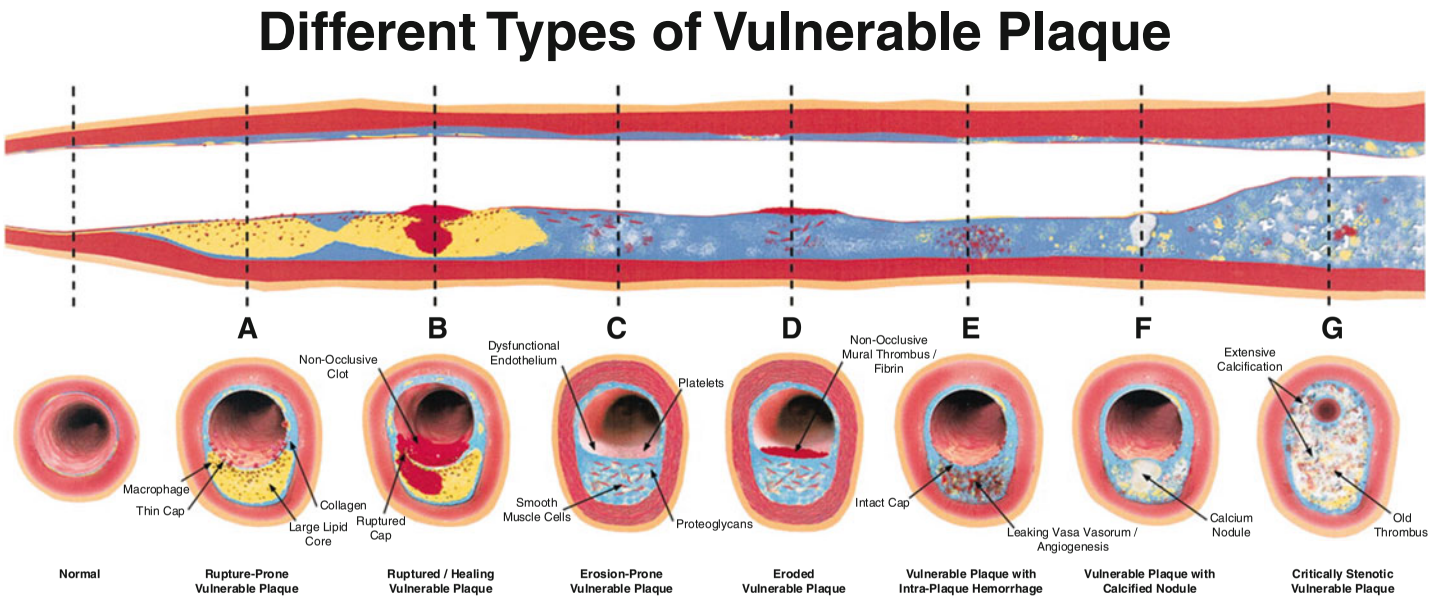


Fig. 2 Different types of vulnerable plaque as underlying cause of acute coronary events (*ACS*) and sudden cardiac death (*SCD*). (**a**) Rupture-prone plaque with large lipid core and thin fibrous cap infiltrated by macrophages. (**b**) Ruptured plaque with subocclusive thrombus and early organization. (**c**) Erosion-prone plaque with proteoglycan matrix in a smooth muscle cell-rich plaque. (**d**) Eroded plaque with subocclusive thrombus. (**e**) Intraplaque hemorrhage secondary to leaking vasa vasorum. (**f**) Calcific nodule protruding into the vessel lumen. (**g**) Chronically stenotic plaque with severe calcification, old thrombus, and eccentric lumen (Reprinted with permission from Naghavi et al. 2003)

In retrospective autopsy studies, three histologic features were more commonly observed in plaques thought to be responsible for most acute coronary events compared with stable plaques: a larger lipid core (>40 % of total lesion area), a thinner fibrous cap (<65 μ), and more inflammatory cells (about 26 % macrophage infiltration of fibrous cap compared with 3 % in stable plaques) (Kolodgie et al. 2004; Virmani et al. 2006). The major criteria to define a vulnerable plaque included active inflammation; a thin cap (<100 μ) with a large lipid core (>40 % of the plaque's total volume); endothelial denudation with superficial platelet aggregation; fissured cap, which may indicate a recent rupture; or severe stenosis, which would make the plaque more prone to shear stress or may be a marker of other less stenotic but vulnerable plaques (Naghavi et al. 2003). According to this proposal, the presence of at least one of these major criteria may indicate a higher risk for plaque complication. The minor criteria for plaque vulnerability included the presence of superficial calcified nodules; yellow color, which may indicate a larger lipid core; intraplaque hemorrhage; endothelial dysfunction (impaired endothelial vasodilator function); and expansive (positive) remodeling, which refers to compensatory outward enlargement of the vessel wall without luminal compromise (Naghavi et al. 2003). Notably, several investigators have noted the presence of more than one vulnerable plaque in patients at risk of cardiovascular events (Libby et al. 2002; Libby 2005; Eriksson 2004; Arbab-Zadeh 2015) underlying the importance of going beyond a vulnerable plaque and called for evaluating the total arterial tree as a whole. In addition, evidence suggests that systemic factors may play a role in plaque instability, including the presence of a systemic inflammatory state (Naghavi et al. 2003). This provides the rationale to studying serum biomarkers that may identify patients with high-risk lesions (vulnerable blood), which, along with vulnerable myocardium, form the triad of vulnerability that defines the vulnerable patient (Naghavi et al. 2003). Indeed, there is no conclusive evidence that individual plaque assessment better predicts acute coronary event risk than established risk factors, such as the extent and severity of coronary artery disease (Arbab-Zadeh 2015; Fig. 3). Current data suggest that rather than focusing on individual coronary arterial lesions, we need a comprehensive, integrative approach for identifying and managing patients at risk of adverse cardiovascular events (Arbab-Zadeh 2015).

Natural History of the Vulnerable Plaque

There are few longitudinal studies that investigated the natural history of plaque features that could be indicative of vulnerability or instability. Such studies involved a baseline imaging evaluation of the morphology of coronary (Motoyama et al. 2009; Kim et al. 2009; Bayturan et al. 2009; Ohtani et al. 2006; Lee et al. 2004) plaques and analyzed the occurrence of clinical events, imaging end points, or both in patients at follow-up. One of the largest studies to date (Motoyama et al. 2009) involved 1,059 patients with suspected or known disease who had computed tomography angiographic examinations and were followed for 27 months for the development of acute coronary syndrome (ACS). The coronary lesions were

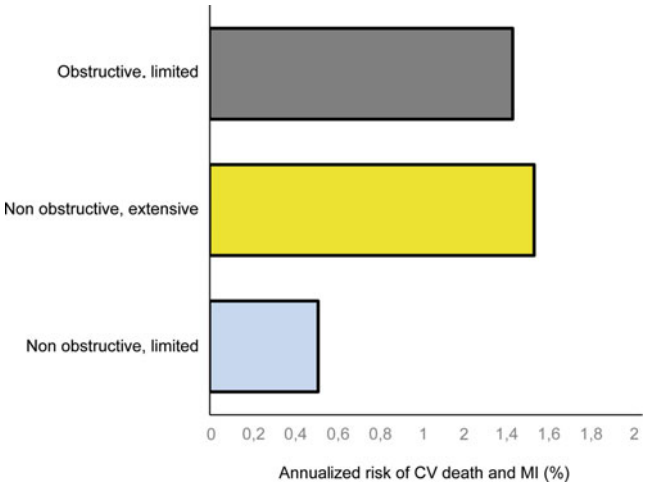
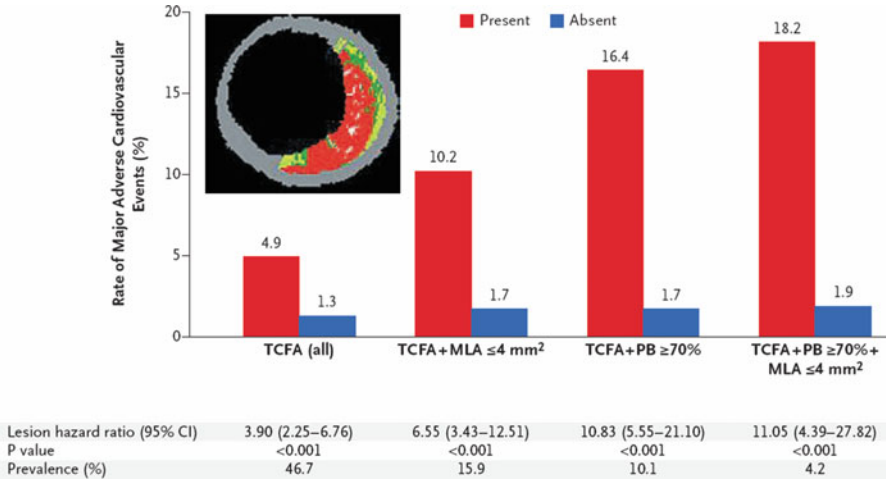


Fig. 3 Annualized risk (percent) of myocardial infarction (*MI*) or cardiovascular (*CV*) death in 3,242 patients followed for a median of 3.6 years after baseline computed tomographic coronary angiography, according to the extent and severity of coronary artery disease. Risk is low in patients with nonobstructive disease (<50 % stenosis) involving four or fewer coronary artery segments (limited disease). Conversely, risk is similarly high in patients with nonobstructive disease if more than four segments are affected (extensive disease) compared with patients with obstructive disease (≥50 % stenosis) (Modified from Arbab-Zadeh et al. 2015)

analyzed for the presence of two features of vulnerability: positive remodeling (>10 % diameter at the plaque site compared with the reference segment) and low attenuation plaques (non-calcified plaque with at low density). An ACS developed in 10 of 45 (22 %) patients that showed plaques with both vulnerability features, compared with 4 of 820 (0.5 %) patients that showed plaques without these features. None of the 167 patients with normal angiography results developed ACS. The presence of 1- or 2-feature positive plaques was the only significant independent predictor of ACS (hazard ratio, 22.8 [95 % CI, 6.9–75.2]) (Motoyama et al. 2009).

The PROSPECT (Providing Regional Observations to Study Predictors of Events in the Coronary Tree) was the first prospective, multicenter study of the natural history of coronary atherosclerosis, using multimodality intravascular imaging to identify the clinical and lesion-related factors that place patients at risk for adverse cardiac events (Stone et al. 2011). In this study, 697 patients with ACS underwent three-vessel coronary angiography and grayscale and radiofrequency intravascular ultrasonographic imaging after percutaneous coronary intervention (PCI). Subsequent major adverse cardiovascular events (MACE) were adjudicated to be related to either originally treated (culprit) lesions or untreated (nonculprit) lesions (Stone et al. 2011). The 3-year cumulative rate of major adverse cardiovascular events was 20.4 %. Events were adjudicated to be related to culprit lesions in 12.9 % of patients and to nonculprit lesions in 11.6 %. Although the nonculprit lesions that led to major adverse cardiovascular events were frequently mild on angiographic assessment, most were characterized by a large plaque burden, a small luminal



Lesion hazard ratio (95% CI)	3.90 (2.25–6.76)	6.55 (3.43–12.51)	10.83 (5.55–21.10)	11.05 (4.39–27.82)
P value	<0.001	<0.001	<0.001	<0.001
Prevalence (%)	46.7	15.9	10.1	4.2

Fig. 4 Event rates in the PROSPECT trial for lesions that were and those that were not thin-cap fibroatheromas, at a median follow-up of 3.4 years (Reprinted with permission from Stone et al. 2011)

area, or both, as seen on grayscale intravascular ultrasonography but not on angiography; no major adverse cardiovascular events arose from untreated segments with a plaque burden resulting in less than 40 % loss of cross-sectional luminal area (Stone et al. 2011). The prospective identification of nonculprit lesions associated with major adverse cardiovascular events was further enhanced by the use of radiofrequency intravascular ultrasonography to characterize the morphologic features of plaques, with thin-cap fibroatheromas (TCFAs) representing the highest-risk phenotype, a finding that is consistent with the established concept of vulnerable plaque. Conversely, major adverse cardiovascular events related to nonculprit lesions rarely developed from non-fibroatheromas, regardless of the plaque burden or minimal luminal area (Stone et al. 2011; Fig. 4).

Vulnerable Plaque Formation

Studies in genetically engineered mice deficient in apolipoprotein E, which develop advanced plaques similar to those in patients, have increased our understanding of certain clinical observations. An increase in T-helper type 1–like lymphocytes promoted TCFAs occurrence, indicating a possible role of the T-helper switch in the formation of these presumably vulnerable plaques.

Some authors (Sluijter et al. 2006) studied matrix metalloproteinases, which can degrade cap constituents, and an extracellular matrix metalloproteinase inducer in carotid endarterectomy specimens. Increased activity of matrix metalloproteinases 8 and 9 was associated with an inflammatory plaque phenotype, and different glycosylation forms of extracellular matrix metalloproteinase inducer were

associated with varying degrees of matrix metalloproteinase activity. It was concluded that extracellular matrix metalloproteinase inducer glycosylation may play a role in plaque destabilization.

Vascular and hemodynamic forces are also likely to play a role in the formation and rupture of TCFAs. Several studies (Stone et al. 2003; Slager et al. 2005; Waxman et al. 2006) demonstrated that areas of low shear stress predispose to the formation of advanced plaques, presumably by creating conditions that favor transmigration of lipids and inflammatory cells into the vessel wall. High shear stress, on the other hand, can promote plaque rupture and platelet aggregability, leading to an occlusive thrombotic event (Stone et al. 2003; Slager et al. 2005; Waxman et al. 2006).

Biomarkers of Vulnerability and Their Potential Application to Prognosis

Increased understanding of the processes causing atherosclerosis has facilitated efforts to identify novel markers of risk that may be circulating in plasma and readily available for sampling.

To date, several biochemical markers have been investigated (Alsheikh-Ali et al. 2010; Seifarth et al. 2014; Battes et al. 2014; Ellims et al. 2014; Puri et al. 2013; Fuchs et al. 2012; Deftereos et al. 2012; Kubo et al. 2009; Hong et al. 2009; Rodriguez-Granillo et al. 2005; Van Mieghem et al. 2005; Drakopoulou et al. 2009; Table 1), C-reactive protein (CRP) and matrix metalloproteinases (MMPs) being the most commonly studied, and their concentrations were most commonly compared with imaging findings of plaques.

C-Reactive Protein

CRP is an acute-phase reactant and nonspecific marker of inflammation, produced predominantly in hepatocytes as a pentamer of identical subunits in response to several cytokines (Norata et al. 2009). Interleukin (IL)-6, one of the most potent drivers of CRP production, is released from activated leukocytes in response to infection or trauma and from vascular smooth muscle cells in response to atherosclerosis. CRP directly binds highly atherogenic oxidized LDL cholesterol and is present within lipid-laden plaques (Libby 2002).

The possible mechanistic role of CRP in plaque deposition is highly complex, exerting pro-atherogenic effects in many cells involved in atherosclerosis (Zhang et al. 1999). CRP may facilitate monocyte adhesion and transmigration into the vessel wall – a critical early step in the atherosclerotic process (Libby et al. 2008). Furthermore, M1 macrophage polarization, catalyzed by CRP, is a proinflammatory trigger in plaque deposition, leading to macrophage infiltration of both adipose tissue and atherosclerotic lesions (Kones 2011). Beyond its role in triggering immunity in plaque deposition, in vitro studies have also shown an association among CRP, inhibition of endothelial nitric oxide synthase, and impaired vasoreactivity 15 and

Table 1 Novel biomarkers for the identification of vulnerable plaque (Modified from Stary (2000))

Biomarker		Diagnosis of ACS	Prognosis	Clinical implication
Fatty acid-binding protein	Ischemia	+	++	
Growth differential factor-15	Ischemia/reperfusion	++	++	+
C-reactive protein	Inflammation: nonspecific marker	++	+++	
Pregnancy-associated plasma protein-A	Inflammation: matrix metalloproteinase-9/plaque instability	+		
Myeloperoxidase	Inflammation: neutrophil activation, reactive oxygen species	+	++	
ST2	Inflammation: regulatory protein in times of myocardial stress	+	+	
Lysosomal phospholipase A2	Cholesterol trafficking	+	++	+
Copeptin	Stress: vasopressin prohormone	+	+	
Soluble CD40 ligand	Platelet activation	+	+	
Fibrinogen	Thrombosis	+	++	
Plasminogen activator inhibitor-1	Endogenous fibrinolytic system	+		
D-dimer	Thrombosis	+	+	
Metabolite profile	Early signs of metabolic dysregulation	+		

ACS acute coronary syndrome, *MI* myocardial infarction, + limited or contradictory evidence, ++ compelling but not conclusive evidence, +++ strong/validated evidence for use

16. An isoform of CRP, monomeric CRP, is stimulated by platelet activation and has prothrombotic and inflammatory properties of its own (Eisenhardt et al. 2009). Monomeric CRP has also been found in plaques, particularly in regions of monocyte-mediated inflammatory activity, and within lipid microdomains of endothelial cells (Ji et al. 2009).

An association of CRP with risk for cardiovascular disease (CVD) has been described in many studies (Musunuru et al. 2008). The Multiple Risk Factor Intervention Trial (MRFIT) was the first of many primary prevention, prospective epidemiological studies to show a strong relationship between levels of CRP and mortality from CVD in high-risk middle-aged men (Kuller et al. 1996). A similar association between increasing CRP levels and subsequent rate of MI and stroke was found in an analysis of apparently healthy men (Ridker et al. 1997).

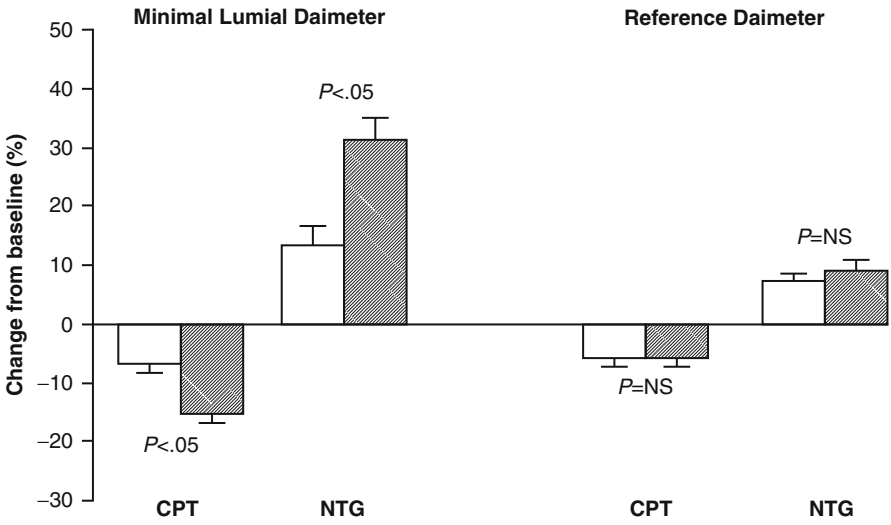


Fig. 5 Percent changes from baseline of minimal lumen diameter (*MLD*) (*left*) and of reference diameter (*RD*) (*right*) in patients with normal (*open bars*) and elevated (*hatched bars*) CRP serum levels during cold pressor test (*CPT*) and after intracoronary injection of nitroglycerin (*NTG*) (Reprinted with permission from Tomai et al. 2001)

CRP has been evaluated extensively also in the setting of stable coronary artery disease (Tomai et al. 2005; Versaci et al. 2000; Gaspardone et al. 1998) and ACS (Morrow et al. 2007; Liuzzo et al. 1994; Biasucci et al. 1999). Elevated levels of CRP at the time of admission have been shown in multiple studies to be associated with poor outcomes in patients with ACS (Morrow et al. 2007; Liuzzo et al. 1994; Biasucci et al. 1999). The strength of that relationship varies depending of the degree of myocardial necrosis, the cut point applied, the timing of measurement, and the patient population (Morrow et al. 2007). Notably, elevated CRP concentrations are independently associated with enhanced vasoreactivity of the culprit lesion, but not in uninvolved epicardial coronary segments (Tomai et al. 2001; Fig. 5), supporting the concept that the increased vasoreactivity is a local plaque-related phenomenon (Tomai 2004). Assessing l*e*vels of CRP several weeks after ACS, when the acute inflammatory phase has subsided, may be more useful than in the acute setting. Patients with a CRP level >2 mg/L 1 month after admission for ACS were at significantly greater risk of death and heart failure (Scirica et al. 2009) compared with those with low levels of CRP.

Recently, the interaction of high-risk nonculprit lesions with CRP levels, which were measured at presentation, 1 month, and 6 months, then categorized at each time as normal (<3 mg/L), elevated (3–10 mg/L), or very elevated (>10 mg/L), has been examined among patients enrolled in the PROSPECT study (Kelly et al. 2014). Patients with elevated CRP levels at any time did not have more high-risk nonculprit lesions; however, untreated high-risk nonculprit lesions were more likely to cause subsequent MACE in patients with very elevated compared with normal 6-month

CRP levels (for thin-cap fibroatheromas, 13.8 % vs. 1.9 %, $p = 0.0003$; for lesions with minimal luminal area $\leq$4.0 mm2, 15.6 % vs. 2.2 %, $p < 0.0001$). As expected, patients with very elevated 6-month CRP levels had higher rates of subsequent nonculprit lesion-related MACE (19.0 % vs. 7.2 %, $p = 0.039$) (Kelly et al. 2014).

Notably, wide variability of CRP levels exists among individuals (Yousuf et al. 2013). The interplay of CRP genetic polymorphisms, influence of genetic loci mediating CRP response, and lifestyle factors contributes to individual, ethnic, and sex-related variation in CRP concentration (Yousuf et al. 2013). A uniform cut point for CRP based on a single value should not be applied universally among all individuals. Body mass index, metabolic syndrome, diabetes mellitus, hypertension, oral contraceptive use, physical exercise, moderate alcohol consumption, periodontal disease, dietary patterns, environmental pollutant burden, and smoking cause significant baseline variation (Yousuf et al. 2013; Kones 2010).

Matrix Metalloproteinases

The MMPs comprise a family of at least 23 active proteinases. MMPs and other proteinases can provoke net destruction of the vascular extracellular matrix in late-stage atherosclerosis, leading to plaque rupture (Galis et al. 1994). Importantly, loss of collagen in the shoulder regions of thin-cap fibroatheromas could reduce tensile strength and precipitate plaque rupture, leading to MI or strokes (Libby 2013). A broad spectrum of MMP inhibitors has been tested in preclinical studies without producing a net effect on atherosclerosis progression or histological features of instability, most likely because of the opposing roles of different MMPs. By contrast, two studies with selective MMP inhibitors showed favorable effects on plaque stability in apolipoprotein E-knockout mice models (Johnson et al. 2011; Quillard et al. 2011). However, translating this data into clinically useful therapies is hampered by the sheer numbers of MMPs and the conflicting results obtained in other animal models (Newby 2015).

Novel Biomarkers

There are several biomarkers reflecting a variety of pathophysiologic pathways that have been reported to be elevated in patients with ACS and potentially associated with increased risk. These include markers of ischemia and inflammation (ischemia-modified albumin, heart fatty acid-binding protein, myeloperoxidase), vascular dysfunction (pregnancy-associated plasma protein A0), biomechanical stress (copeptin, ST2, growth differentiation factor [GDF]-15), hemostasis (fibrinogen, plasminogen activator inhibitor-1), and lipid metabolism (lipoprotein-associated phospholipase A2) (Scirica 2010; Ferrante et al. 2010). Few of the novel biomarkers have been shown to consistently improve on established markers, and many lack confirmation in varied cohorts. In a study of 664 patients admitted with suspected

ACS, for example, none of the more than ten novel markers tested approached the sensitivity of cardiac troponin in diagnosing MI (McCann et al. 2008).

Several authors have proposed analytical and clinical criteria that novel biomarkers must successfully meet before they can be fully integrated into clinical care (Jaffe et al. 2006). Of the novel markers, GDF-15, a member of the transforming growth factor family that is released by myocytes during ischemia and reperfusion, is one of the most promising. In several cohorts (Eggers et al. 2008; Wollert et al. 2007), elevated levels of GDF-15 are associated with increased risk of death and MI, independent of ECG changes, troponin level, or NP level. In one study, there was an interaction between randomization to an invasive strategy and elevated levels of GDF-15, which suggests that an invasive strategy may be preferential in patients with an increased concentration (Wollert et al. 2007), although prospective confirmatory studies are needed.

Proteomics, Metabolomics, Genomics, and Pharmacogenomics

Advances in proteomic, metabolic, and genomic profiling with high-throughput screening technology combined with advanced bioinformatic and statistical techniques may dramatically expand the number of novel markers of cardiac metabolism and pathology. For example, a study of serial blood samples from patients undergoing alcohol septal ablation, in other words a "planned MI," revealed a specific profile of metabolites in pyrimidine metabolism, the tricarboxylic acid cycle, and the pentose phosphate pathway that were present within 10 min of the induced MI. The pattern was also present in patients with ACS undergoing PCI but not in patients with stable CAD undergoing PCI (Lewis et al. 2008). Genome-wide association studies, which evaluate hundreds of thousands of single nucleotide polymorphisms, have identified several potential variants such as those at chromosome 9p21 that are associated with an increased risk of incident CVD (Samani et al. 2007). Further studies are needed to determine whether individuals with single nucleotide polymorphisms at chromosome 9p21 are also at increased risk of secondary events after ACS.

Conclusions

In the quest for individualized medicine, biomarkers have emerged as a tool for improved risk prediction.

An ideal biomarker should demonstrate quantitative differences in patients with and without disease. Further, it should have predictive value in prospective studies and incremental benefit over standard clinical risk markers. The goal of measuring a biomarker should not only be risk assessment but rather ascertaining information that would alter the threshold of the pretest risk to change clinical management in a cost-effective manner. The ideal risk marker should demonstrate these features with rigorous evidence and independence (Hlatky et al. 2009).

During the last two decades, a number of biomarkers have been considered in the assessment of coronary plaque composition identifying the risk for primary and secondary prevention of cardiovascular diseases. However, further research is needed to undoubtedly determining a candidate that materially adds to established models of risk assessment and modification.

Summary Points

- Substantial research has been recently conducted in order to develop new methods to identify subjects at risk before the occurrence of a cardiovascular event.
- The concept of "vulnerable plaque" has gained attention as a paradigm to improve risk stratification and potentially lead to the discovery of novel markers of risk to prevent cardiovascular disease.
- Biochemical markers have been investigated for the identification of coronary atherosclerotic plaque composition and early detection of their vulnerability.
- C-reactive protein and matrix metalloproteinases are the most commonly studied, but also novel biomarkers reflecting a variety of pathophysiologic pathways have been reported to be potentially associated with increased risk of coronary events.

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Part V

Functional and Structural Variables

Pulse Pressure and Pulse Pressure Amplification as Biomarkers in Cardiovascular Disease

39

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_7

Abstract

Recent evidence indicated that pulse pressure and pulse pressure amplification, the ratio or difference between the peripheral and central pulse pressure, might provide prognostic information in patients with cardiovascular diseases. Theoretically, any emerging clinical biomarker should be easy in application, reliable in measurement, predictable in prognosis, and instructive in treatment. Herein, in this chapter, we will focus on the measurement, reference, prognosis, and treatment of pulse pressure and pulse pressure amplification and expound them as biomarkers in cardiovascular disease.

Keywords

Biomarker • Pulse pressure • Pulse pressure amplification • Measurement • Reference • Prognosis • Treatment

Abbreviations

ACE	Angiotensin-converting enzyme
ARB	Angiotensin receptor blocker
AUC	Area under curve
CI	Confidence interval
CKD	Chronic kidney disease
HR	Hazard ratio
SD	Standard deviation
WHO	World Health Organization

Key Facts of Pulse Pressure and Pulse Pressure Amplification

- Pulse pressure is considered as a cardiovascular biomarker since 1990s.
- Pulse pressure amplification, an emerging cardiovascular biomarker, is the ratio or difference between peripheral and central blood pressure.
- Pulse pressure amplification is practical for clinical use, with reliable measurement and established reference system.
- Prognostic value of pulse pressure amplification is proved in various populations, especially in the elderly.
- Angiotensin-converting enzyme inhibitor and angiotensin receptor blocker and calcium channel blocker are effective agents in increasing pulse pressure amplification.

Definitions

Pulse pressure Pulse pressure is a blood pressure component, which is calculated as the difference between systolic and diastolic blood pressure.

Pulse pressure amplification Pulse pressure amplification is considered as a novel cardiovascular biomarker, which is calculated as the ratio or difference between the peripheral and central pulse pressure.

Tonometry-based device Central blood pressure can be measured noninvasively by tonometry-based device, such as SphygmoCor and PulsePen, with applanation tonometry for pulse waveform recording and calibration by brachial blood pressure.

Reference value Reference value contains the one- or two-tail cutoff values, derived from the large-scale measurements, which is used by physicians to identify the abnormal cases in clinical practice.

Prognostic value A biomarker with prognostic value means it can be used to predict prognosis, such as future mortality and events.

Introduction

High blood pressure is the most common and important cardiovascular risk factor and is considered a global public crisis. On April 7, 2013, Professor Margaret Chan, the director of the World Health Organization (WHO), demonstrated that hypertension affected more than one billion people worldwide and led to over nine million deaths per year (WHO report 2013).

In history, as early as the nineteenth century, Riva-Rocci introduced the sphygmomanometer in clinical practice, the first device for assessing arterial blood pressure (Riva-Rocci 1896). During the following century, attention focused on the extreme values of systolic and diastolic blood pressure recorded at the brachial artery. However, diastolic blood pressure fell by the wayside as a predictor, when Franklin SS et al. proved that an elevated diastolic blood pressure lost its prognostic value in subjects over 50 years old from the Framingham study (Franklin et al. 2001). Furthermore, in elderly patients, Franklin SS et al. also indicated that diastolic blood pressure was inversely related to cardiovascular risk (Franklin et al. 1999). Before Franklin, brachial mean blood pressure, together with pulse pressure, made a strong showing as a risk predictor (Darne et al. 1989) but was overtaken by pulse pressure as the best pressure indicator (Sesso et al. 2000; Thomas et al. 2001; Miura et al. 2001; Lewington et al. 2002).

More recently, some studies highlighted the importance of central systolic blood pressure and central pulse pressure as cardiovascular prognostic factors. In theory, central blood pressure is superior to peripheral blood pressure, as a reliable indicator of blood pressure, since it is the real pressure imposed on the left ventricle. In this respect, central blood pressure measurement is of great interest in terms of the clinical application, and some devices for the noninvasive central blood pressure measurement, such as SphygmoCor, were developed (Williams et al. 2006; Waddell

et al. 2001). Moreover, Jankowski et al. provided invasive evidence favoring central over peripheral pulse pressure for risk prediction (Jankowski et al. 2008).

Normally, central blood pressure is lower than peripheral blood pressure, so the difference between peripheral and central blood pressure should be a positive value, known as blood pressure amplification (Nijdam et al. 2008). Moreover, Safar ME et al. and Benetos A et al. all indicated that the disappearance of the blood pressure amplification phenomenon (the lower blood pressure amplification) was a significant predictor of all-cause and cardiovascular mortality, independent of age and other standard confounding factors (Safar et al. 2009; Benetos et al. 2010). Later, other clinical investigations further indicated that the absence of pulse pressure amplification is a significant predictor of cardiovascular mortality in the general population and in the elderly (Benetos et al. 2012; Cho et al. 2013). For instance, in more than 1,100 nursing-home residents over the age of 80 years from the PARTAGE study, it was indicated that reduced pulse pressure amplification was significantly and independently associated with the presence of cardiovascular diseases and was a strong predictor of total and cardiovascular mortality (Benetos et al. 2012).

Theoretically, any emerging biomarker, such as pulse pressure amplification, should be easy in application, reliable in measurement, predictable in prognosis, and instructive in treatment, and it should also provide complementary and independent prognostic value compared with existing biomarkers. In this chapter, we will expound pulse pressure and pulse pressure amplification as new biomarkers in cardiovascular disease.

Pulse Pressure as a Biomarker in Cardiovascular Disease

Pulse pressure, the difference between systolic and diastolic blood pressure, is considered as a reliable indicator of arterial stiffness and as a biomarker of asymptomatic target organ damage, especially in the geriatric population. In history, many clinical investigations indicated the significant association of cardiovascular events and mortality with pulse pressure, and we summarized the major prospective data in Table 1.

In 1994, Madhavan et al. indicated that in 2207 hypertensives, a wide pretreatment pulse pressure was significantly associated with subsequent cardiovascular complications, and the extreme value of diastolic blood pressure, either too high or too low, would lead to a great risk of myocardial infarction, after adjustment for sex, race, age, and previous cardiovascular disease (Madhavan et al. 1994). Fang J et al. indicated that in 5730 hypertensives, after a follow-up of over 5 years, pulse pressure was significantly associated with myocardial infarctions in both untreated patients and all patients, with hazard ratios (HRs) of 1.49 (95 % confidence interval [CI] 1.18–1.89) and of 1.72 (1.47–2.01), respectively (Fang et al. 1995). In 1997, Benetos A. indicated that in 19083 Frenchmen

Table 1 Major prospective investigations on the association of cardiovascular end points with pulse pressure

Investigator, year	Subjects (mean age, years)	Events and mortality	Major findings (hazard ratio (95 % confidence interval))
Madhavan et al. (1994)	2, 207 hypertensives	MI and cardiovascular mortality	A wide pretreatment pulse pressure was associated with subsequent cardiovascular complications in hypertensives
Fang et al. (1995)	5, 730 hypertensives (53)	MI	Pulse pressure was significantly associated with the occurrence of MI in all subjects (1.74 (1.41–2.01))
Benetos et al. (1997)	19, 083 Frenchmen (40–69)	All-cause and cardiovascular mortality	A wide pulse pressure was an independent significant predictor of all-cause, especially coronary mortality
Franklin et al. (1999)	1, 924 subjects (50–79) (Framingham Heart Study)	Coronary heart disease	PP (1.23 (1.16–1.30)) was better than SBP (1.16 (1.11–1.21)) or DBP (1.14 (1.03–1.26)) in predicting CHD risk
Thomas et al. (2008)	69,989 subjects (>50)	Cardiovascular stroke and coronary mortality	Increased PP predicts cardiovascular mortality, acting more on coronary than cerebral vessels

MI myocardial infraction, *PP* pulse pressure, *DBP* diastolic blood pressure, *CHD* coronary heart disease

aged 40–69 years, pulse pressure was an independent and significant predictor of cardiovascular and all-cause mortality (Benetos et al. 1997). The most convincing evidence was from the Framingham Heart Study with over 20-year follow-up, in which 1924 subjects between 50 and 79 years of age with no clinical evidence of coronary heart disease and free of antihypertensive treatment (Franklin et al. 1999). In this study, Franklin SS et al. concluded that higher pulse pressure was a critical indicator of cardiovascular risk, and pulse pressure was superior to systolic and diastolic blood pressure in predicting coronary heart disease with a HR of 1.23 (1.16–1.30) per 10 mmHg.

With those solid evidences, pulse pressure is considered as a critical risk predictor and an asymptomatic target organ damage in cardiovascular disease, especially in patients over 50 years old. Although pulse pressure over 60 mmHg was considered as an asymptomatic target organ damage according to the guideline from the European Society of Hypertension, as far as we know, there is still no clinical trial focusing on pulse pressure control as primary treatment target. Further studies or post hoc analyses are warranted in this field.

Pulse Pressure Amplification as a Biomarker in Cardiovascular Disease

Basic Concept of Central Blood Pressure and Pulse Pressure Amplification

Central blood pressure is the blood pressure in the ascending aorta (Salvi 2012). Many years ago, central blood pressure could only be measured by the invasive method, using catheter-based BP monitor. Nowadays, with the development of tonometry technique and pulse wave analysis, it can be measured noninvasively with tonometry-based devices, and the methodology was validated by the invasive measurement (Papaioannou et al. 2009). From a physiological viewpoint, during the systole, central blood pressure is the pressure that the left ventricle directly confronts, so it affects cardiac afterload and cardiac work and is the main contributor in the development of left ventricular remodeling. During the diastole, central blood pressure influences the coronary blood flow and maintains an adequate subendocardial perfusion (Salvi 2012). So in summary, central blood pressure defines the cardiac work in the systole, whereas in the diastole, it affects the regular blood flow to the ventricular myocardium. However, central blood pressure is pressure dependent or calibration dependent, so, more recently, the ratio of peripheral and central blood pressure, which is independent of pressure measurement or calibration procedure, known as blood pressure amplification, is recognized as a better pressure indicator (Avolio et al. 2009). Then, pulse pressure amplification, the ratio of peripheral and central pulse pressure, is proved as a potential biomarker for arterial stiffness, especially in the geriatric population (Benetos et al. 2012).

Measurements of Central Blood Pressure and Pulse Pressure Amplification

As shown in Fig. 1, peripheral pressure waveform (right panel) is noninvasively recorded by tonometry device, and it is calibrated by the brachial systolic and diastolic blood pressure or the diastolic and mean blood pressure, which are assessed by the brachial blood pressure monitor. Then, the aortic pressure waveform can be transformed by the peripheral pressure waveform via a validated transfer function. This generalized transfer function is derived by applying several mathematical techniques (e.g., time domain or frequency domain analysis) and validated by several clinical investigations. Alternatively (left panel) the central pressure waveform can be directly recorded on carotid artery by tonometry-based devices and then calibrated by the mean and diastolic brachial blood pressure in order to obtain central systolic blood pressure and pulse pressure, since the mean and

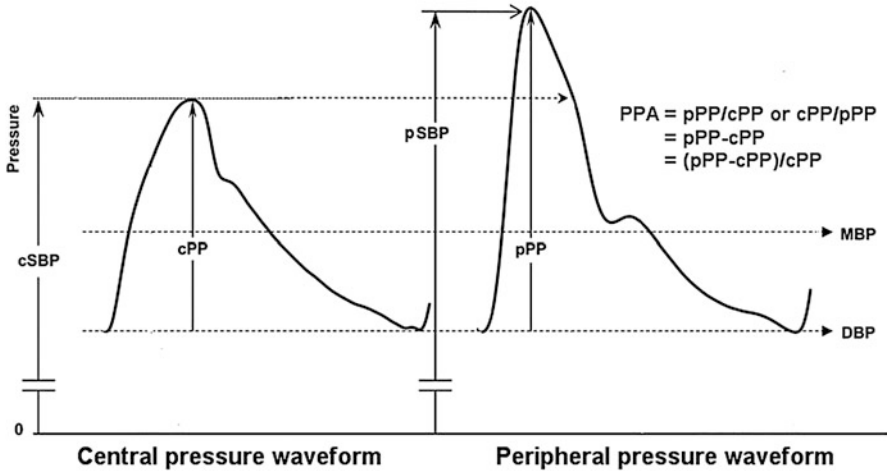

Fig. 1 Measurements on central blood pressure and pulse pressure amplification. Peripheral pressure waveform (*right panel*) is firstly recorded and calibrated by the brachial systolic and diastolic blood pressure. Peripheral mean and diastolic blood pressure are calculated with the area under curve (AUC) method. Central pressure waveform (*left panel*) is recorded on the carotid artery or transformed by the peripheral pressure waveform via a validated transfer function. Central pressure waveform can be calibrated by the mean and diastolic blood pressure in order to obtain central systolic blood pressure and pulse pressure, since the mean and diastolic blood pressure almost remain unaltered in the entire arterial tree. Pulse pressure amplification can be calculated by the ratio of peripheral and central pulse pressure, or the difference in mmHg between peripheral and central pulse pressure, or the difference divided by the central pulse pressure. *pSBP* peripheral systolic blood pressure, *pPP* peripheral pulse pressure, *cSBP* central systolic blood pressure, *cPP* central pulse pressure, *MBP* mean blood pressure, *DBP* diastolic blood pressure, *PPA* pulse pressure amplification

diastolic blood pressure almost remain unaltered in the entire arterial tree (Avolio et al. 2009).

The superiority of the two methodologies (direct carotid recording versus the use of the transfer function) is still under debate.

It is well established that the blood pressure differs markedly between peripheral (brachial) and central arteries (aorta). As the pressure wave travels distally from the heart, a gradual and significant increase of systolic blood pressure and pulse pressure occurs. This phenomenon is called blood pressure amplification and is under extensive investigation, especially the pulse pressure amplification (Avolio et al. 2009). In previous investigations, pulse pressure amplification was calculated by several formulas. Most commonly, it is defined by the ratio of peripheral and central pulse pressure, as indicated in Fig. 1. Alternatively, it can also be expressed as the difference (in mmHg) between peripheral and central pulse pressure or the difference divided by the central pulse pressure (Fig. 1) (McEniery 2008; Segers et al. 2009).

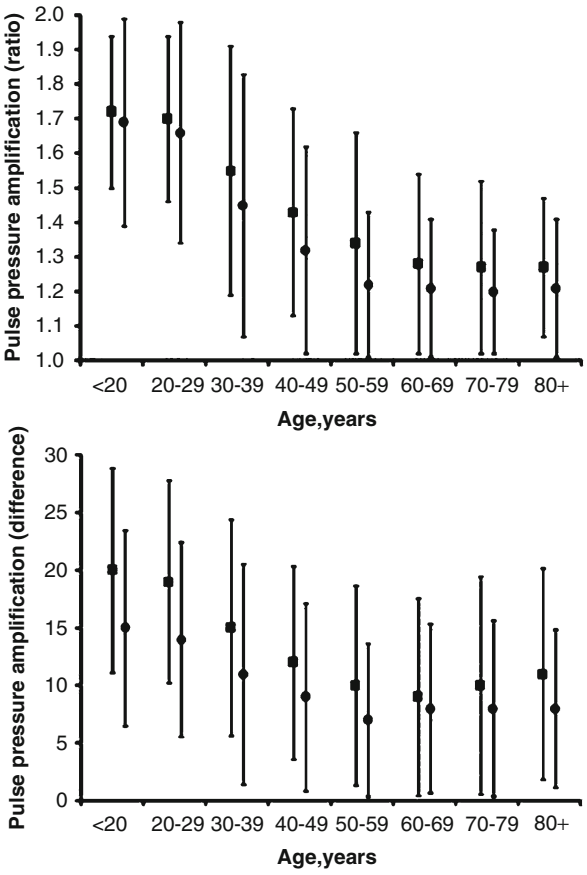

Fig. 2 Reference value of pulse pressure amplification according to age group in men and women. The mean pulse pressure amplification and 95 % confidential interval are expressed as the ratio of peripheral and central pulse pressure (*upper panel*) and the difference between peripheral and central pulse pressure (*lower panel*). Men are indicated as the filled square, and women are indicated as the filled circle (Adapted from McEniery 2008, with permission)

Reference Value of Pulse Pressure Amplification

In the literature, limited data is available regarding the reference value of pulse pressure amplification. In the Anglo-Cardiff Collaborative Trial (ACCT), central blood pressure was determined by the radial pressure waveform with the help of the validated transfer function and calibrated by the brachial systolic and diastolic blood pressure in 5648 participants, and pulse pressure amplification is calculated by the ratio of peripheral and central pulse pressure and by the difference between them. Pulse pressure amplification, expressed by the ratio of peripheral and central pulse pressure, varied from about 1.7 in subjects <20 years old to about 1.2 in subjects >80 years old. The corresponding values for the absolute difference between peripheral and central pulse pressure were 20 mmHg for subjects <20 years old and 7 mmHg for subjects >80 years old (Fig. 2) (McEniery 2008). Recently, a meta-analysis involved 45, 436 subjects with measurements of pulse pressure

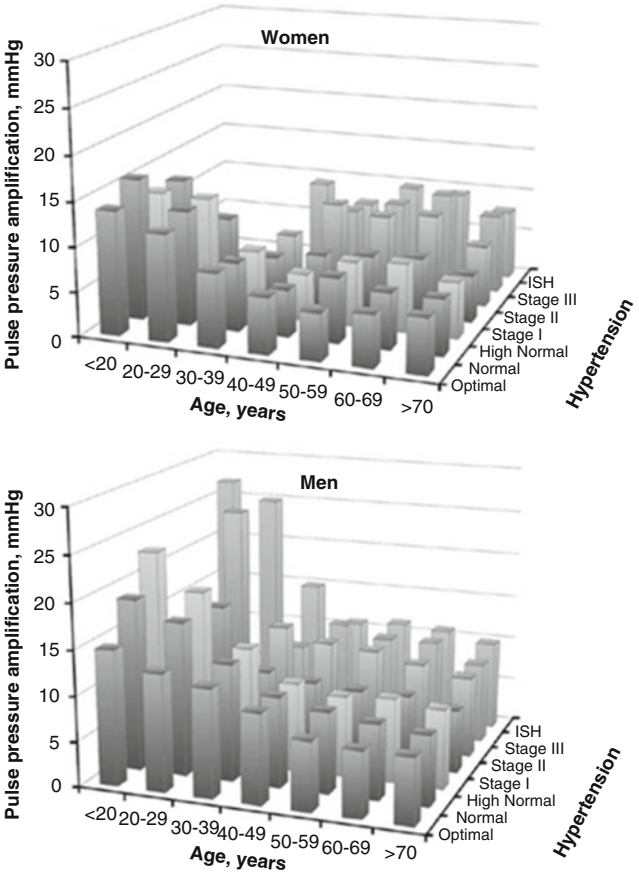

Fig. 3 Pulse pressure amplification stratified by blood pressure category and age in both men and women. Pulse pressure amplification is presented according to age groups and blood pressure categories (optimal blood pressure, normal blood pressure, high normal blood pressure, stage I hypertension, stage II hypertension, stage III hypertension, isolated systolic hypertension). Pulse pressure amplification is calculated as the difference between peripheral and central pulse pressure. *ISH* isolated systolic hypertension (Adapted from Herbert et al. 2014, with permission)

amplification from 77 studies, and most subjects are apparently healthy, without antihypertensive or anti-dyslipidemia therapy and free of overt cardiovascular disease and diabetes (Herbert et al. 2014). As shown in Fig. 3, pulse pressure amplification was stratified by blood pressure category and age in both men and women. It is noteworthy that pulse pressure amplification gradually decreases with age, and the magnitude is greater in men than in women. Moreover, at the similar age and blood pressure level, men had averagely 6.6 mmHg greater pulse pressure amplification than women.

Influencing Factors of Pulse Pressure Amplification

The determinants of pulse pressure amplification are still unclear, or its clinical relevance is still under debate. Cross-sectional data in healthy subjects from the ACCT study (McEniery 2008) and the Asklepios study (Segers et al. 2009) showed that pulse pressure amplification is modulated by vascular properties, such as large artery stiffness, peripheral resistance, and mainly pressure wave reflections, as well as by heart rate. The principal mechanism of these factors influencing pulse pressure amplification largely relied on the "timing–synchronization" of the forward and reflected pressure waves. In addition, classical non-modifiable (i.e., age and sex) and modifiable cardiovascular risk factors (i.e., high blood pressure, high plasma glucose, hypercholesterolemia, and smoking) or established cardiovascular disease are also significantly associated with reduced pulse pressure amplification in observational studies (Wilkinson et al. 2001; McEniery 2005). These factors may accelerate biological vascular ageing, which is per se the main modulator of large artery stiffness and wave reflections.

From this point of view, pulse pressure amplification, integrating other cardiovascular risk factors and global arterial properties, could serve as a biomarker of cardiovascular risk (Benetos et al. 2012). The available data imply that pulse pressure amplification is not just a mathematical expression but carries additional physiological information, potentially above that of central and peripheral blood pressure alone.

Prognostic Value of Pulse Pressure Amplification

In the literature, most prospective data indicated that pulse pressure amplification, expressed by the ratio or the difference between the peripheral and central pulse pressure, was significantly associated with cardiovascular events and mortality. As shown in Table 2, in 2008, Nijdam ME et al. indicated that in men between 40 and 80 years of age, a higher pulse pressure amplification was significantly associated with a better cardiovascular risk profile, a reduced pulse wave velocity, a reduced common carotid intima-media thickness, and a lower Framingham risk score of coronary heart disease, after adjustment for age, blood pressure level, body height, and heart rate (Nijdam et al. 2008). However, in 2010, in general population from the Framingham Heart Study, pulse pressure amplification failed to provide independent predictive information for major cardiovascular events (HR, 0.86 [0.19, 3.82]) (Mitchell et al. 2010). On the contrary, Benetos A et al. indicated that in a large French cohort at a mean age of 40.4 years old ($n = 125, 151$), 1 standard deviation (SD) increase in brachial pulse pressure was significantly associated with cardiovascular and all-cause mortality, with HRs of 1.17 and 1.13, respectively; the corresponding HRs for the estimated carotid pulse pressure were 1.20 and 1.17, respectively, while the pulse pressure amplification exhibited the highest HRs as 1.30 and 1.19 for cardiovascular and all-cause mortality, respectively (Benetos et al. 2010).

Table 2 Major investigations on the association of cardiovascular outcomes with pulse pressure amplification

Investigator, year	Participants (mean age, years)	Measurement of PPA	Events and mortality	Major findings
Nijdam et al. (2008)	400 men (40–80)	bPP/cPP	10-year risk of CHD using Framingham score	A higher PPA reflected a lower CV risk in men between 40 and 80 years of age
Benetos (2010)	125, 151 Frenchmen (40.4)	Estimated cPP/bPP	All-cause and CV mortality	PPA was a strong risk predictor with a HR of 1.22 and 1.41 for CV and all-cause mortality, respectively
Mitchell et al. (2010)	2,232 patients (63 ± 12) (Framingham Heart Study)	bPP/cPP	CV events	PPA was not significantly associated with CV events ($P = 0.84$)
Benetos et al. (2012)	1, 126 patients in nursing home (88 ± 5) (PARTAGE study)	(bPP-cPP)/cPP	All-cause mortality major CV events	A 10 % increase in PPA was associated with a 24 % decrease in total mortality and a 17 % decrease in major CV events
Regnault et al. (2012)	72, 437 men (41 ± 11) 52, 714 women (39.5 ± 11.6)	bPP/cPP	Age-related CV mortality	In postmenopausal women, PPA contributed to the significant increase in CV risk
Cho et al. (2013)	80 patients undergoing CAG (62.7 ± 10.1)	cPP/bPP	Extent of CHD	PPA was related to the severity of CAD, particularly in patients <65 years old
Wassertheureu et al. (2014)	135 patients with CKD 2 to 4 (60 ± 14.9)	bPP/cPP	Renal end points all-cause mortality	Patients with CKD stage 4 and low PPA had the highest risk for renal end points, adjusted for age and proteinuria

PPA pulse pressure amplification, *bPP* brachial pulse pressure, *cPP* central pulse pressure, *CV* cardiovascular, *CAD* coronary angiograph, *CHD* coronary heart disease, *CKD* chronic kidney disease

The most convincing data were derived from the PARTAGE study, a longitudinal study with a mean follow-up of 2 years, in which 1126 elderly subjects over 80 years old, living in the nursing home, were included (Benetos et al. 2012). In this study, Benetos A et al. indicated that a 10 % increase in pulse pressure amplification was significantly and independently associated with a 24 % decrease in total mortality

and a 17 % decrease in major cardiovascular events, after adjustment for other potential confounders. Regnault V et al. also found that pulse pressure amplification was highly predictive of differences in the age-related cardiovascular mortality in men and women, separately, after adjustment for known cardiovascular risk factors (Regnault et al. 2012).

Moreover, some investigators also reported that pulse pressure amplification was also a significant predictor of severity of coronary heart disease in patients undergoing coronary angiograph. For instance, Cho SW et al. (Cho et al. 2013) indicated that after adjustment for known risk factors, pulse pressure amplification was significantly related to the severity (evaluated by the Gensini score) of coronary heart disease. In addition, Wassertheurer S et al. assessed pulse pressure amplification in 135 patients with chronic kidney disease (CKD) stage 2 to 4 and 89 controls, in which pulse pressure amplification was reduced in CKD patients as compared with the control and significantly and independently associated with the decline in renal function and mortality, after adjustment for age and proteinuria (Wassertheurer et al. 2014).

In summary, pulse pressure amplification, expressed by the ratio or difference between peripheral and central pulse pressure, predicts cardiovascular events and mortality in most studies, especially in the elderly. Assessment of this parameter could help in risk assessment and improve diagnostic and therapeutic strategies in those patients.

Pulse Pressure Amplification and Treatment

Although it seems well established that pulse pressure amplification is a significant predictor of cardiovascular events and mortality, data are scarce regarding the effect of cardiovascular agents on it. In Table 3, major investigations in this field with regard to principal cardiovascular agents, such as adrenoceptor-β blocker, calcium channel blocker, angiotensin-converting enzyme (ACE) inhibitor, and angiotensin receptor blocker (ARB), were summarized.

As to adrenoceptor-β blocker, the first direct evidence came from a subgroup analysis in the REASON study ($n = 354$) (Asmar et al. 2001). In this study, Asmar RG et al. indicated that after a 12-month treatment, atenolol exhibited a more pronounced antihypertensive effect on peripheral blood pressure than central blood pressure, and, consequently, pulse pressure amplification was significantly lower in the atenolol treatment arm, as compared with placebo. Similarly, in a small-sample, randomized, double-blinded study in untreated hypertensives at middle age, Dhakam et al. also indicated that pulse pressure amplification was significantly reduced after 6 weeks of the atenolol treatment (Dhakam et al. 2006).

London G et al. investigated the long-term antihypertensive effect of nitrendipine on peripheral and central blood pressure, in 24 patients with end-stage renal disease. Data indicated that nitrendipine significantly reduced both peripheral and central blood pressure (London et al. 1994). However, the effect on central pulse pressure was more prominent than peripheral pulse pressure,

Table 3 Major investigations on treatment of pulse pressure amplification

Agent, dosage	Comparator	Investigator, year	Duration	Participants (mean age, years)	Measurement of PPA	PPA		
						Agent	Comparator	*P*
Adrenoceptor-β blockers								
Atenolol, 5 mg	Baseline	Asmar et al. (2001)	12 months	354 untreated hypertensives (53)	bPP/cPP	1.09	1.22	<0.001
Atenolol, 5 mg	Baseline	Dhakam et al. (2006)	6 weeks	21 untreated hypertensives (51)	bPP/cPP	1.21	1.38	<0.001
Atenolol, 5 mg	Placebo	Dhakam et al. (2008)	5 weeks	16 low-risk uncontrolled hypertensives (70)	bPP/cPP	1.2	1.39	<0.001
Nebivolol, 5 mg								
Calcium channel blockers								
Nitrendipine, 20/40 mg	Baseline	London et al. (1994)	12 months	24 patients with ESRD (53)	bPP/cPP	1.13	1	<0.01
Angiotensin-converting enzyme inhibitor and angiotensin receptor blocker								
Perindopril, 2/4 mg tiw	Baseline	London et al. (1994)	12 months	24 patients with ESRD (53)	bPP/cPP	1.1	1.02	<0.01
Eprosartan, 400 mg	Baseline	Dhakam et al. (2006)	6 weeks	21 untreated hypertensives (51)	bPP/cPP	1.42	1.38	<0.001
Quinapril, 20 mg	Placebo	Aznaouridis et al. (2007)	2 h	100 hypertensives (53)	bPP/cPP	9.8 ± 4.4	8.8 ± 7.6	NS
Captopril, 25 mg						11.4 ± 5.7		
Telmisartan, 80 mg						11.4 ± 5.7		

PPA pulse pressure amplification, *bPP* brachial pulse pressure, *cPP* central pulse pressure, *ESRD* end-stage renal dysfunction, *NS* nonsignificant

and pulse pressure amplification was therefore significantly increased after a 12-month nitrendipine treatment.

In the literature, ACE inhibitor and ARB are more extensively studied. In a randomized and double-blind clinical investigation with placebo run in and two parallel active treatment groups, London et al. indicated that in 24 patients with end-stage renal disease, perindopril significantly reduced patients' pulse pressure amplification after a 12-month treatment (London et al. 1994). Similarly, Dhakam et al. also reported that in 21 untreated hypertensives (mean age, 51 years), eprosartan significantly reduced peripheral and central blood pressure but significantly increased patients' pulse pressure amplification (Dhakam 2006). Aznaouridis K et al. (Aznaouridis et al. 2007) also investigated the transient antihypertensive effect of ACE inhibitor and ARB on pulse pressure amplification, namely, captopril 25 mg and quinapril 20 mg and telmisartan 80 mg, but without significant change.

In summary, clinical studies favor calcium channel blocker, ACE inhibitor, and ARB in terms of pulse pressure amplification increment. However, adrenoceptor-β blocker, mainly atenolol, decreases pulse pressure amplification, which may be largely attributed to the associated bradycardia and the consequent resynchronization of the reflected pressure wave relatively earlier in the systolic phase.

Conclusion

Pulse pressure has been recognized as an established cardiovascular biomarker for decades and was proved in the Framingham Heart Study. Recent data indicated that pulse pressure amplification, the ratio or difference between peripheral and central pulse pressure, might provide prognostic value in patients with cardiovascular diseases, especially in the elderly. Normally, it requires at least four characteristics for any emerging biomarker to be a clinical practical one, namely high-reproducibility measurement, reference for clinical use, incremental prognostic value, and guidance in treatment. Pulse pressure amplification, a pressure-independent parameter reflecting patients' arterial stiffness and other cardiovascular risks, could be reproducibly measured by the noninvasive tonometry-based device, and the reference value has been set to screen for the abnormal in clinical practice. Most population studies and clinical data indicated that pulse pressure amplification could provide independent prognostic value for cardiovascular and all-cause mortality and other renal and cardiac outcomes. In treatment, ACE inhibitor and ARB and calcium channel blocker are effective in increasing pulse pressure amplification, whereas adrenoceptor-β blocker may act in the opposite direction. In summary, pulse pressure amplification is an emerging biomarker in cardiovascular disease but is still on the way to be a reliable and practical one. Further studies are still warranted to ensure the incremental prognostic value of pulse pressure amplification in various populations. Besides, whether the increase in pulse pressure amplification by cardiovascular agents can eventually result in patients' prognostic

benefit, it is still uncertain and is the most important issue to be proved in future investigations.

Potential Applications to Prognosis, Other Diseases, or Conditions

Pulse pressure, an established cardiovascular biomarker, indicates the severity of patients' arterial stiffness and is considered as an asymptomatic target organ damage in various populations, especially those over 50 years old. Pulse pressure amplification, another emerging indicator of arterial stiffness and a pressure-independent index, potentially provide incremental prognostic information over known cardiovascular risk factors. However, controversy exists in the literature. In general population, such as in the Framingham Heart Study, pulse pressure amplification failed to provide independent predictive value for cardiovascular and all-cause mortality. On the contrary, in the geriatric population, like the PARTAGE population, pulse pressure amplification served as a strong and independent death predictor. It is hypothesized that pulse pressure amplification, like pulse pressure, favors the elderly and high-risk population, with regard to the death and event prediction. Further studies are still warranted to prove the incremental prognostic significance of pulse pressure amplification and enlarge its clinical application.

Summary Points

- Pulse pressure is an established biomarker in cardiovascular disease, especially in patients over 50 years old.
- Pulse pressure amplification, a pressure-independent biomarker, can be reproducibly measured by noninvasive tonometry-based devices, and its reference has been set for clinical use.
- Pulse pressure amplification, integrating other cardiovascular risk and global arterial properties, is a cardiovascular biomarker.
- Pulse pressure amplification acts as an independent predictor of cardiovascular and all-cause mortality and other renal and cardiac outcomes.
- Angiotensin-converting enzyme inhibitor and angiotensin receptor blocker and calcium channel blocker increase pulse pressure amplification, but adrenoceptor-β blocker deceases.

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Ventricular Activation Time as a Marker for Diastolic Dysfunction

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_45

Abstract

Hypertension in the developed world affects up to 30 % of adults causing a significant disease burden. Poorly controlled hypertension precipitates structural changes resulting in heart failure. Reports suggest that diastolic left ventricular dysfunction may be the earliest detectable sequence that may precede left ventricular hypertrophy clinically detected by the standard 12-lead electrocardiogram voltage criteria.

Early diagnosis of hypertension, focusing on diastolic dysfunction parameters by electrocardiogram, is of clinical value, but not clinically well established yet. Only few studies have investigated atrial changes, reflected by P wave voltage/duration abnormalities, in diastolic dysfunction patients. These were criticized by the low sensitivity to disease severity and pathophysiology identification. Hence, the need for new electrical markers was urged.

As early as 1982, the close relationship between the delays in the time for ventricular depolarization "called ventricular activation time" (known also as intrinsicoid deflection) and left atrial (LA) abnormalities was documented in spontaneous hypertensive rat models. However, we know little about similar changes in humans.

Ventricular activation time, measured in milliseconds on the surface electrocardiogram from the onset of the QRS complex to the peak of the R wave (QR interval), has provided a new marker in predicting diastolic dysfunction. The ventricular activation time prolongation in diastolic dysfunction patients, without left ventricular hypertrophy, has proven association and is proportionally increased with diastolic dysfunction progression (from grade I to grade III). Other studies have examined the direct relationship between diastolic dysfunction and atrial changes presented by relatively novel electrocardiogram P wave markers (P wave terminal force in lead V1 and P wave dispersions). We therefore reviewed the available evidence of novel interval electrocardiogram markers in newly diagnosed hypertensive patients with evidence for diastolic dysfunction.

Keywords

Ventricular activation time • P wave dispersions • P wave terminal force in V1 • Diastolic dysfunction and hypertension

Abbreviations

A wave	Late diastolic (atrial contraction)
ASE	American Society of Echocardiography
AVN	Atrioventricular node
BNP	B-natriuretic peptide

CVA	Cerebrovascular accidents
DD	Diastolic dysfunction
E wave	Early diastolic
H-V	His-ventricle conduction
IHD	Ischemic heart disease
LA	Left atrium
LV	Left ventricle
LVH	Left ventricular hypertrophy
LVMI	Left ventricle mass index
PJK	Purkinje fibers (system)
PTFV1	P wave terminal force in V1
PWD	P wave dispersions
RV	Right ventricle
SAN	Sinoatrial node
TDI	Tissue Doppler image
TMD	Transmitral Doppler
VAT	Ventricular activation time

Key Facts of Electrocardiogram Interval and Ventricular Activation Time, P Wave Terminal Force in Lead V1, and P Wave Dispersions

- Ventricular activation time (VAT) is the time between the beginnings of the QRS deflection to the peak R. It is believed to be in the range of 35–40 ms.
- The earliest exit for ventricular activation is in the mid-right ventricle cavity and then distal part of the septum. While the difference in right and left ventricular voltages on the surface ECG is subtle (the whole septum is completely activated in 0.015 s), the QR interval presents the combination of both right and left ventricle activation.
- VAT (or intrinsicoid deflection) is a marker for left ventricular hypertrophy if more than 50 ms and epicardial origin of ventricular tachycardia if more that 85 ms.
- VAT prolongation is not always associated with left ventricle hypertrophy.
- Further ECG analysis depicted an evidence in P wave terminal force in V1 (PTFV1) which is defined as the multiplication of the P wave terminal negative deflection amplitude in V1 (i.e., each small square equals to 1 mm or 0.1 mv) and duration (ms). A negative cutoff value of P wave terminal forces more than and/or equal to 40 mm.ms is considered significant (PTFV1).
- PTFV1 predominantly represents changes in left atrial electrical propagation. The combination of voltage and duration is superior to voltage parameters alone. PTFV1 was appreciated in many studies as a prognostic marker for cardiovascular morbidity and mortality.

- P wave dispersion (PWD) is defined as the difference between the longest and shortest P wave duration, in 12-lead ECG, in milliseconds.
- Longer PWD durations showed a good correlation with the parameters of impaired diastolic function.

Key Facts About Diastolic Dysfunction

- Diastolic heart failure is defined as the signs and/or symptoms of heart failure with preserved left ventricular systolic function according to the guideline task force definition. It is also defined as heart failure with preserved ejection fraction (HFPEF).
- The diastolic phase is composed of four phases, isovolumic relaxation, early diastole, and diastasis and atrial contraction phases. Diastolic heart failure can occur as a result of many heart muscle diseases.
- The common etiologies include hypertension, aortic valve disease, cardiomyopathy, systolic heart failure, and older age group.
- Echocardiography is the investigation of choice used to assess the LV diastolic parameters in all cardiac society guidelines.
- The parameters of echocardiographic assessment of diastolic function include the following: transmitral Doppler early diastolic deceleration of the mitral valve (E wave), late diastolic declaration (A wave), E/A ratio, tissue Doppler images (TDI) of mitral valve annulus e prime (E′), A prime (A′), and E′/A′. Also isovolumic relaxation time (IVRT), deceleration time (DT) of MV cusp, and pulmonary flow systolic/diastolic (S/D ratio) ratios are also valid parameters in the guidelines.
- According to the above parameters, diastolic dysfunction is classified as impaired relaxation (stage I), pseudonormal (stage 2), and restrictive pattern (stage 3).

Definitions

Diastolic heart failure (diastolic dysfunction) It reflects signs and symptoms of heart failure with preserved left ventricular systolic function (i.e., ejection fraction greater than 50 %).

Diastolic phase This is the cardiac relaxation phase and is composed of isovolumic relaxation, early diastole, diastasis and atrial contraction phases of the cardiac cycle.

Left ventricular stiffness index It is calculated by dividing the E/E′ (i.e., transmitral Doppler "early mitral valve deceleration E wave" and tissue Doppler "early diastole E′") by the left ventricular end-diastolic dimension.

P wave dispersion (PWD) It is defined as the differences between the longest and shortest P wave duration, in 12-lead ECG, in milliseconds.

P wave terminal force in V1 (PTFV1) It is defined as the multiplication of P wave terminal negative deflection amplitude in V1 (i.e., each small square equals to 1 mm or 0.1 mv) and duration (ms). The negative cutoff value of P wave terminal forces more than and/or equal to 40 mm.ms was considered positive (PTFV1).

Ventricular activation time (VAT) It is defined as the ventricular conduction time of electrical signals from the His bundle to PJK system (known also as intrinsicoid deflection). It represents the QR interval on the surface ECG preferably on pericardial leads.

Introduction

Hypertension is one of the leading causes of morbidity and mortality worldwide. Untreated hypertension leads to adverse and concealed effects on the myocardium that precede disease diagnosis. Left ventricular diastolic dysfunction is an early common association in hypertension that may take place even without clear evidence of electrocardiogram (ECG) abnormalities. Conventional electrocardiogram voltage criteria for left ventricular hypertrophy (LVH) represent long-standing secondary damage to the myocardium.

Hence, there is a need to find novel predictive markers for hypertensive diastolic dysfunction. Detailed analysis of the 12-lead electrocardiogram may highlight the new markers in suspected diastolic heart failure. Atrial wall electrical remodeling, as presented by P wave dispersions and P wave terminal force in lead V1, may provide early evidence of diastolic impairment. However, ventricular activation time (VAT), which represents the myocardium excitation time for pulse transmission from His fibers to Purkinje fibers, has shown a consistent relation with the diastolic dysfunction and has a proportional progression through disease stages (Boles et al. 2010). These may represent an early and easy tool for early diagnosis of hypertension that, in turn, would enhance an earlier detection of diastolic dysfunction (DD) and treatment before disease progression.

This review covers the aspects of cardiac electrical system in relation to diastolic heart disease and the evidences of new ECG diagnostic markers.

Cardiac Conduction System

The cardiac electrical conduction system can auto-generate and transmits regular impulse independently to optimize cardiac output and stroke volume according to various physiological circumstances, i.e., exercise and stress. This feature is fundamentally maintained by the unique structure of the conducting system maintaining the balance between the sympathetic and the parasympathetic systems.

To ensure this dynamic activity, the heart has an automated cells located specifically at the sinoatrial (SA) node, atrioventricular node (AV node), His bundle, and Purkinje fibers. As the premium cardiac pacemaker, the initiated SA node impulse

propagates through the atrium; subsequently the impulse travels to the ventricle through the AV node, His bundle, and Purkinje (PKJ) system fibers. This stable and fast pulse propagation provides the point of maximum excitation that does not have a decremented prolongation as seen in AV node conduction.

Fundamental Basics of Action Potential and Impulse Excitation

Electrical impulse is generated through the excitation of the cardiac cell membrane. Cardiac cells in resting state have high intracellular K^+ approximately 30 times more than extracellular concentrations due to high K^+ permeability. In contrast intracellular Na^+ concentration is 30 times less. Due to this resting transmembranous gradient, a magnitude polarization is generated which is measured at −90 mv. This ensures a fundamental change of ion membrane permeability that is followed by phases of dynamic transmembrane action potential mediated by Na^+, K^+, and Ca^{++}. There are four different phases to return to the pre-excitement phase. In the excitement phase 0 (the upstroke), the sudden change in Na+ permeability leads to a higher intracellular Na+ level that triggers a reverse in the transmembrane potential to 20+ mv. Immediately afterward, phases 1 and 2 took place where there is reduction of Na+ and K+ permeability and hardly any ion exchange through the membrane resulting in a plateau phase and the potential remains around 0 mv. Subsequently, with the efflux of the intracellular K^+ into the extracellular fluid, repolarized phase 3 ensues promptly. Resting potential period represents phase 4 until the subsequent wave of excitation arrives. As a result of the change of the potential variation across the membrane, a propagation of impulse is initiated leading to a series of currents across the membrane in the forward direction of excitation of the next adjacent segment of the myocardial fiber.

Electrocardiogram: The Sequence of Excitation

The electrocardiogram (ECG) fundamentally relays the anatomical and the electrical distribution of the cardiac impulse in a tightly coordinated and synchronized electrical activity within the myocardium. On the ECG, the P wave represents a depolarization of the atrial muscle fibers through Bachman's bundle, which is essentially preceded by the invisible initial activity (on the surface ECG) of the pacemaker cells at the SA node. The subsequent propagation from the atrium through the AV node and the mid-His bundle is illustrated in the PR interval. This is followed by the ST segment, which represents the plateau of the ventricular potential and the succeeding ventricular repolarization (referred to as T wave). As a result of this tightly coordinated electrical activity, a synchronized contraction of the myocardial fibers is generated on the surface ECG wave and is timely relevant to the heart muscle activation.

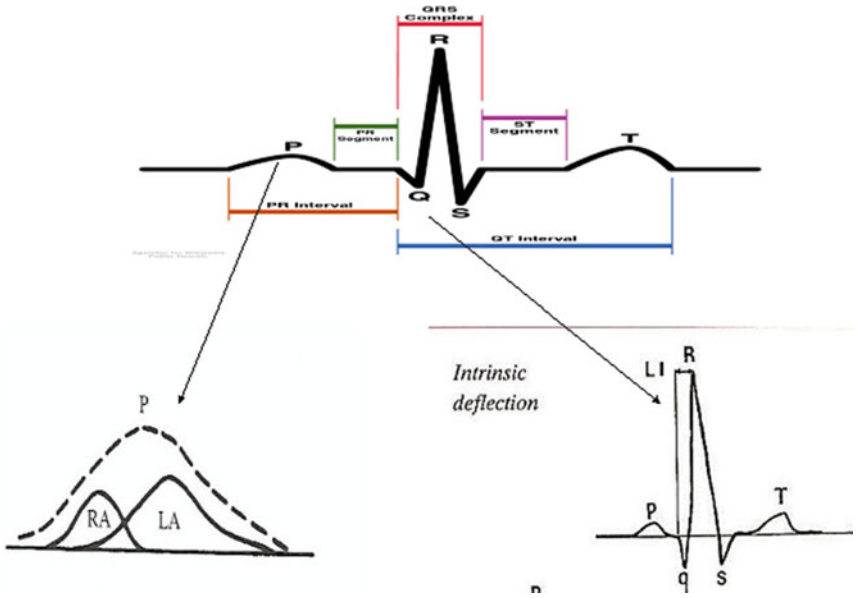

Fig. 1 This figure explains both P wave morphology and VAT duration (represents QR interval)

In fact the P wave reflects the transition of the electrical impulse from the right to the left atrium generating two different activities in lead V1, whereas the VAT is constant with the period from the onset of the first deflection on ECG of QRS duration to the peak of the R wave on pericardial leads (Fig. 1). The VAT is referred to as (His-ventricle) interval as well, which reflects the time from the His bundle activation to the brisk exit from the (PJK) system to the myocardium at once to complete ventricular activation. In fact the earliest exit of ventricular impulse is in the mid-right ventricle (the earliest exit from the right bundle tract), yet the delay between right and left bundles into PJK is almost negligible in intact bundles. However, it is evident that complete activation time may prolong with increased cardiac muscle mass that is relevant to left ventricular hypertrophy where the left ventricular mass is increased. Yet the new concept of early electrical remodeling has been investigated to explain prolonged ventricular activation in normal anatomical myocardial thickness (Bacharova et al. 2010).

Ventricular Activation Time: Propagation and Interpretation

Activation starts on the left side of the septum from 0.01 to 0.015 s earlier than the right side. However, since the left-side branch of the His bundle enters the septum higher than the right-side branch, the septum is thicker on the left side, and the earliest output on the right side is mid-RV cavity that facilitates faster activation on the right septum, and the earliest output direction of the vector is essentially to the

right mid-cavity. This first wave of electric movement is rather important as it writes the normal septal Q wave in leads aVL, V6, and I. This Q wave initiates the QRS complex in leads II, aVR wave, and I (Netter 2005).

The cardiac apex depolarizes immediately after the right ventricle (RV) depolarization as a result of a second wave of electrical impulse which reflects the R wave on surface ECG on II, III, and I. The right ventricular depolarization occurs quickly and completes earlier than the left ventricular owing to the thinness of the RV muscle structure compared to that of the left ventricle (LV) (Netter 2005). The third wave is the spread of the depolarization toward the lateral wall of LV and coincides with R wave amplitude in II and I and S wave in lead III. The left ventricular delayed depolarization occurs as the result of the fourth wave spread toward the base and left ventricle and occurs just before the end of the ventricular depolarization process, and it would be reflected on surface ECG as deep S wave in lead III and R wave in leads II and I. This would conclude the whole process and the ventricle repolarizes gradually in this refractory state as the ST wave.

Having understood the above basic electro-structural physiology concepts, we can gradually develop an understanding of the pathological ECG ventricular activation duration changes in certain cardiac conditions like diastolic heart failure, which is our focus in this chapter (Boles et al. 2010).

Diastolic Heart Dysfunction

The disease is recognized as symptoms and/or signs of heart failure with preserved systolic function. The latter is calculated by dividing the stroke volume by the end-diastolic volume, which is expected to be high when there is increased resistance to filling of the LV in diastolic heart failure. Hence, a failure of the left ventricle to relax in the diastolic phase of the cardiac cycle is commonly associated with systolic dysfunction at the same time. However, DD is defined in another way as heart failure signs or symptoms with preserved ventricular systolic function.

The diastolic phase is composed of four phases known as isovolumic relaxation, early diastole, and diastasis and atrial contraction phases. Diastolic heart failure may be associated with a systemic disease or local myocardial pathology (Table 1).

Since the diastolic phase is the only chance for LV filling, the changes in diastolic parameters may lead to significant hemodynamic disturbances. These changes of cardiac cycle time intervals are diagnostic and are used to diagnose and grade the severity of the disease by comprehensive echocardiograpic study.

Epidemiology and Clinical Burden of Diastolic Heart Failure

It is estimated that at least 15–20 million patients in the 51 European states have diastolic heart failure. The disease burden is growing and it represents a major clinical challenge. T Kuznetsova et al. estimated the prevalence of the diastolic heart failure among all presentations with heart failure as high as 27.3 %, and it was

Table 1 Diastolic dysfunction etiologies according to disease stage and pathophysiology (ESC guidelines, Eur J Heart Fail. 2008 Oct; 10 (10): 933–989)

Impaired relaxation	Reduced compliance
Senile (age-related)	Hypertension (preserved LV systolic function)
Cardiomyopathy • (Familial or genetic involvement) • Dilated per partum cardiomyopathy • Drugs: calcium channel blockers, B blockers, cytotoxic and antiarrhythmic drugs	Myocardial Fibrosis • Previous myocardial infarction • Nutritional causes; like thiamine and selenium deficiency • Infiltrative myocardial involvement: Chag's disease, Sarcoidosis, amyloidosis, hemochromatosis, HIV and end stage renal failure • Endocrinal causes: Diabetes mellitus, hypothyroidism, Cushing syndrome, adrenal insufficiency and phaeochromocytoma
Ischemia related to coronary artery disease	Collage Composition changes
	Restrictive Cardiomyopathy; Infiltrative disease "as above" Constrictive pericarditis

adversely affected by age based on echocardiography indices and confirmed by high NT-pro-BNP level. The reported prevalence in general population varies from 11 % to 34 %. This was influenced by various factors. Older age (above 70 years old) represents 49 % of the cohort and is affected by grade II (pseudonormal) diastolic dysfunction, while the younger age group between 50 and 60 had early diastolic abnormalities (impaired relaxation, i.e., grade I). Myocardial stiffness is progressive with the age advance and hence impaired relaxation and elevated diastolic pressure with age advance (Kuznetsova et al. 2009).

Diastolic heart failure may result in a high level of clinical disabilities and exercise restraint. Moreover, according to Redfield et al.'s Kaplan–Meier curve, the prognosis (morbidity, hospital admissions, and mortality) worsens with the increasing severity of the disease. The mortality rate secondary to mild diastolic impairment was 10 % in a 5-year period compared to 25 % in moderate to severe diastolic dysfunction (grades II and III) (Redfield et al. 2003).

Diagnosis Criteria for Diastolic Dysfunction

Echocardiography is the mainstay for diagnosis diastolic dysfunction using pulmonary flow, parameters of mitral valve Doppler, and tissue Doppler of the mitral annulus, parameters that are validated and continuously updated in all available guidelines. However, invasive cardiac catheterization to assess the elevation in left atrial pressure, end-diastolic pressure, and pulmonary capillary wedge pressure is limited to refractory heart failure patients where both pathophysiologic and prognostic explanations are required. Yet, echocardiography remains a noninvasive

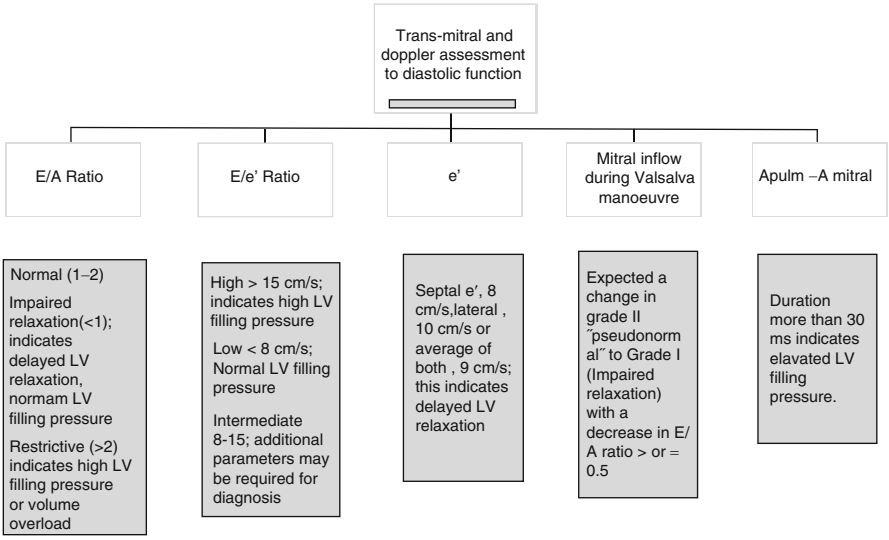

Fig. 2 Diagnostic parameters for transmitral and Doppler echocardiography in diastolic dysfunction. *E/A ratio* the ratio between early E and late A diastolic mitral inflow; *e′* early diastolic velocity of mitral annulus, *E/e′* indicates the ratio of mitral inflow E wave to the tissue Doppler e′. A pulmonary – A mitral represents the time difference between pulmonary vein A wave flow duration to mitral A wave duration (ESC guidelines, Eur J Heart Fail. 2008 Oct; 10 (10): 933–989)

investigation that may offer superior results in the majority of cases (Dickstein et al. 2008).

The American Society of Echocardiography (ASE), the European Association of Echocardiography (EAE), and the British Society of Echocardiography (BSE) have collaboratively established guidelines for the criteria of diagnosis and assessment of diastolic function. The transmitral Doppler parameters (E wave, i.e., early diastole; A wave, i.e., late diastole; or atrial contraction phase and the ratio between them E/A) and tissue Doppler parameters (septal e′, septal a′, e′/a′, and the ratio E/e′) are widely approved parameters to assess diastolic parameters (Fig. 2) (Dickstein et al. 2008; Anguita et al. 2012).

Different modalities of echocardiography are used for the assessment of diastolic function. Structural remodeling changes secondary to certain pathology of diastolic dysfunction are diagnosed by two-dimensional echo, i.e., left ventricular hypertrophy (LVH) secondary to hypertension, valvular disease, or hereditary cardiomyopathy. Doppler study is effective in the quantitative hemodynamic effect at the level of the mitral valve and pulmonary veins. Finally, tissue Doppler imaging adds more weight to the assessment, as it is independent from pre- and post-load pressure effects across the mitral valve. The latter can unmask the pseudonormal pattern of diastolic dysfunction.

The association of impaired LV filling and the rise in the left atrial (LA) pressure has been studied, and its negative morbidity effect on the general population is well established. This relationship between the left ventricular and left atrial remodeling (measured by echocardiography) has deleterious hemodynamic impact on the functional capacity (Redfield et al. 2003).

Clinical Significance of Ventricular Activation Time

Ventricular activation time (VAT) is defined as the ventricular conduction time of electrical depolarization from the His bundle to PJK system (known also as intrinsicoid deflection). As previously mentioned, it simply represents the QR interval on the surface ECG preferably on pericardial leads V5 and V6 (Boles et al. 2010) (Fig. 1).

VAT in V5 or V6 of >0.05 s is clinically employed as one of the Romhilt–Estes scoring criteria for the diagnosis of left ventricular hypertrophy (LVH) (Romhilt and Estes 1968). Moreover, VAT has another clinical significance in the diagnosis of the origin of ventricular tachycardia, i.e., intrinsicoid deflection time (VAT) of $\geq$85 ms indicates an epicardial origin. This has revealed a sensitivity of 87 % and a specificity of 90 % (Berruezo et al. 2004).

Myocardial remodeling leading to LVH is investigated by ECG voltage criteria commonly Sokolow–Lyon and Cornell ECG criteria. While it has been conceived that LVH may lead to a frequent association with broader QRS duration, another interesting study has revealed that increasing the left ventricular mass in LVH does not proportionally correlate with QRS duration or with conduction velocity delays including intrinsicoid deflection. This mismatched relationship between LV mass index and conduction velocity would suggest a different culprit for slower conduction velocity. The concept of electrical remodeling may offer a valid explanation and can occur in apparently normal LV anatomy even before detecting geometrical changes (Bacharova et al. 2010).

However, the close relationship between the delay in the time for the ventricle to depolarize (VAT) (i.e., electrical remodeling) and left atrial (LA) abnormalities (as a marker for diastolic dysfunction) was observed in spontaneous hypertensive rat models in the 1980s, though this relationship has not yet been documented in humans (Kleber et al. 1982).

Almuntaser et al. have demonstrated an interesting finding in the patients with early hypertension and diastolic dysfunction. They investigated voltage-guided ECG criteria against left ventricular stiffness index (by dividing the E/E′ Doppler parameters by the left ventricular end-diastolic dimension). The latter represents a pressure–volume relationship in diastolic dysfunction and relies on the parameters of diastolic dysfunction as per the Canadian guidelines. The result has validated ventricular activation time delay, in apparently normal myocardium (i.e., normal left ventricular mass index and no valvular lesions), as the only ECG markers to left ventricular stiffness in diastolic dysfunction. The study has confirmed progressive VAT prolongation from grade I to grade III of diastolic dysfunction. Additionally,

this study investigated VAT in the LVH group with interventricular septum more than 1.2 cm. While Sokolow, Cornell, and the other five ECG voltage criteria demonstrated a low sensitivity for LVH (2–17 % depends on the voltage criteria used), conversely ventricular activation time was more sensitive (Almuntaser et al. 2007a).

Changes noted on the ECG that are associated with diastolic dysfunction (DD) remain poorly defined. While DD has been well studied in LVH patients, diastolic abnormalities without LVH geometrical changes have not been previously studied in human. Another project conducted by Boles et al. assessed the ventricular activation time (VAT) as a potential marker for DD in early hypertension without myocardial remodeling involvement, i.e., LVH. The extension of this study has focused on relatively novel atrial voltage criteria against DD echo parameters. This is detailed in the subsequent text.

P Wave Terminal Force in V1

P wave terminal force in V1 (PTFV1) has emerged as a new cardiovascular marker that holds a valuable prognostic value (Liu et al. 2013). It is defined as the multiply of P wave terminal negative deflection amplitude in V1 (i.e., each small square equals to 1 mm or 0.1 mv) and duration (ms). A negative cutoff value of P wave terminal forces more than and/or equal to 40 mm.ms was considered positive (PTFV1) (Fig. 3).

Various studies had investigated the relationship between the P wave changes on surface ECG and the cardiovascular events. In particular, P wave terminal force in lead V1 (PTFV1) was studied against the end point of cardiac death or hospitalization for the heart failure. PTFV1 (i.e., combined P duration and voltage) is superior to P wave duration only as a prognostic marker (Liu et al. 2013). In another observation, PTFV1 was found to be proportionally associated with increasing risk

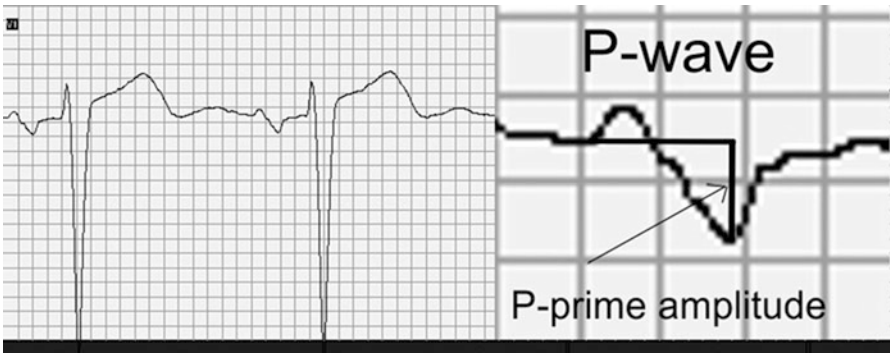

Fig. 3 This figure depicts PTFV1 measurement; the beginning of the P wave serves as the start point of P wave amplitude (Permission approved by Larisa G. Tereshchenko; J Am Heart Assoc. 2014 Dec; 3(6): e001387)

of atrial fibrillation (Soliman et al. 2009). Furthermore, Kohsaka et al. have found a proportional relationship between the presence of PTFV1 and ischemic cerebrovascular (stroke) events. The study has concluded that PTFV1 is a valid predictor to CVA events (Kohsaka et al. 2005). Another large US population study identified PTFV1 as an ECG marker that is associated with increased risk of all-cause, cerebrovascular disease (CVD) and ischemic heart disease (IHD) mortality. PTFV1 could be recommended as a cardiovascular marker for early risk stratification and monitoring disease progression and subsequently for monitoring the response to preventive management (Tereshchenko et al. 2014).

The pathophysiologic explanation of the effects of hypertension links the diastolic dysfunction with left atrial pressure changes resulting from elevated left ventricular end-diastolic pressures. These changes, in turn, are transmitted to the left atrium (LA) leading to continuous stretching and scar formation. In another explanation, atrial changes mainly occur secondary to pressure tension transmitted to the atrial walls from increased resistance in the early diastolic filling phase. Subsequently the remodeled and geometrically changed LA may impede the propagation of the electrical impulse leading to voltage and conduction time augmentation.

The P wave terminal force more than −40 mm.ms in V1 would theoretically reflect LA geometric changes in DD due to delayed electrical propagation in LA. P wave amplitude and duration together (in PTFV1) have a superior diagnostic value with diastolic dysfunction than duration dispersions only. This was confirmed by angiographic findings validating DD against surface ECG in the work of Lee et al. (2005).

Hence PTFV1, as an ECG marker, was studied with DD parameters assessed by echocardiography. Echo parameters for DD were statistically significant with PTFV1 $\geq$40 mm.ms achieving sensitivity of 62 % and specificity of 75 %. This may represent an easy tool in the diagnosis of DD (Boles et al. 2007).

P Wave Dispersions

P wave dispersion (PWD) is defined as the differences between the longest and shortest P wave duration, in 12-lead ECG, in milliseconds. PWD has been extensively studied with varieties of cardiovascular and noncardiac conditions. In DD, Dogan et al. studied PWD in two different groups of patients according to existence of diastolic dysfunction. It was found that the rise of end-diastolic pressure hindered the ventricular filling patterns leading to diastolic abnormalities. This mechanism affects atrial pressure indirectly contributing to the heterogeneous pattern of the atrial wave's propagation in hypertensive patients and, hence, the variations in minimum and maximum P wave duration documented by the electrocardiogram. Longer PWD duration correlates significantly with the parameters of impaired diastolic function (Dogan et al. 2003). Direct explanation is again due to the heterogeneous changes in the left atrial wall leading to interrupted propagation of intra- and interatrial sinus impulses.

At present, PWD is established as a noninvasive marker for atrial fibrillation risk (Dilaveris et al. 2000). PWD has been investigated in many other cardiac and noncardiac diseases, which are beyond the scope of this chapter.

Update in Ventricular Activation Time and P Wave Markers in Diastolic Dysfunction

A recent prospective study was designed in patients with newly diagnosed and untreated hypertension to investigate the VAT and P wave morphology/duration in hypertensive diastolic dysfunction. All patients had a high-resolution ECG and echocardiographic assessment equipped with Doppler tissue echocardiography capabilities (Boles et al. 2010). Baseline echocardiography examinations were done to rule out structural abnormalities, and cardiac dimensions were calculated using the standard M mode (Nagueh et al. 1997). Also, the left ventricular mass index (LVMI) was measured based on the American Society of Echocardiography's guidelines (Lang et al. 2005); subjects with an LVMI that exceeded 115 g/m2 (male) or 95 g/m2 (female) were excluded (i.e., LVH) (Devereux et al. 1986). Left ventricular diastolic dysfunction was assessed using echocardiography parameters according to the consensus guidelines of AHA/ESC task force guidelines (Rakowski et al. 1996; Almuntaser et al. 2007b; van Heerebeek et al. 2006).

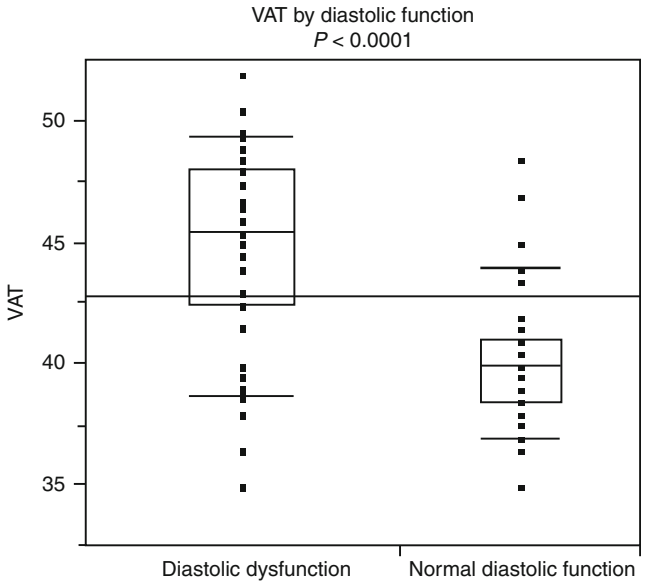

Fig. 4 Ventricular activation time in ms in normal diastolic function and diastolic dysfunction. $P < 0.05$

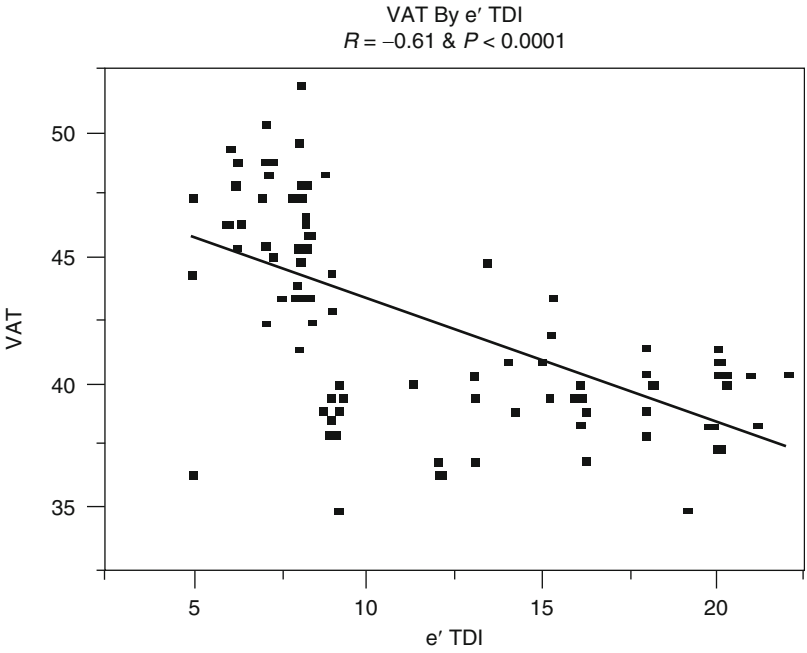

Fig. 5 Relationship between ventricular activation time (VAT) and e′ tissue Doppler imaging (TDI)

Table 2 Data for the covariates and their coefficients, 95 % CI in stepwise regression analysis

	r^2 Change	SE	β	P
VAT, adjusted $r^2 = 0.40$, $P < 0.0001$				
TDI e′/a′ (cm/s)	31	476.785	−4.4265	<0.0001
E/e′	4	53.4103	0.2927	0.0379

TDI tissue Doppler image, *VAT* ventricular activation time

VAT was prolonged in subjects with DD (46.3 ± 0.4 vs. 39.6 ± 0.3 ms, $P < 0.01$) (Fig. 4). This prolongation was statistically significant and proportional to tissue Doppler imaging (TDI) indices as follows: (early diastolic velocity) e′ ($r = -0.53$, $P < 0.0001$; Fig. 5), (ratio of early and late diastolic velocities) e′/a′ ($r = -0.53$, $P < 0.0001$), transmitral Doppler (TMD) (early peak filling rate and early deceleration peak) E/A ($r = -0.32$, $P = 0.001$), and (ratio of early diastolic mitral inflow and early diastolic velocities) E/e′ ($r = 0.44$, $P < 0.0001$).

Multivariate stepwise regression model (Table 2) showed tissue Doppler e′/a′ and E/e′ were independent determinants of VAT in assessing DD without contribution from age, gender, LA dimension, LV mass index, and interventricular septal diameter as covariates ($r^2 = 0.40$, $P < 0.0001$). The best VAT correlations were found in V6, and this may offer a simple approach for screening patients with early diastolic

heart dysfunction even in general practice and at primary care level (Boles et al. 2010).

Another extended (same settings, recruiting a larger cohort of "106 patients") study, by the same group of investigators, focused on changes in P wave morphology and duration, reflecting LA pressure changes occurring with diastolic dysfunction, namely, PTFV1 and PWD.

They drew the conclusion that both PTFV1 and PWD were higher [-43 ± 1.8 mm.ms and 43 ± 2 ms] in subjects with diastolic dysfunction compared to normal diastolic function [-36 ± 1.3 mm.ms and 36 ± 1 ms, $P < 0.005$ and 0.02, respectively]. Moreover, PTFV1 and PWD also showed significant correlation with echo diastolic parameters as TDI E′/A′ [$r = -0.35$, $p < 0.0001$ and $r = -0.37$, $p < 0.0002$] and TMD E/A [$r = -0.24$, $p < 0.01$ and $r = -0.23$, $p < 0.002$]. A cutoff value of PTFV1 $\geq$–40 mm.ms showed sensitivity of 62 %, specificity of 75 %, and positive predictive value of 78 % for diagnosis of diastolic dysfunction (Boles et al. 2007).

Potential Application of ECG Markers in Diastolic Dysfunction

Diastolic heart failure has different structural changes and pathophysiology from systolic heart failure (Devereux et al. 1986). This is related in part to the different disease etiology and pathophysiology but is part of a spectrum of disease with common associations between systolic and diastolic dysfunction frequently documented. Ventricular DD as an early complication of hypertension ensues before the detection of the left ventricular hypertrophy (LVH) by conventional electrocardiogram (Gerdts et al. 2004; Phillips et al. 1989; Smith et al. 1985). Remarkably, the well-known LVH voltage criteria caused by long-standing high blood pressure cannot independently identify diastolic abnormalities (Palmieri et al. 2006). However, careful examination of electrocardiogram atrial parameters, without LVH criteria, can predict diastolic dysfunction providing new diagnostic markers to this common disease.

Electrical cardiac remodeling may be associated with early diastolic heart dysfunction (i.e., velocity variations), and it can precede any increase to the LV mass and the development of LVH in undiagnosed hypertension. Reports in late 1980s and early 1990s supported that DD occurred early in the course of hypertension and precedes measurable LVH (Phillips et al. 1989).

VAT may increase secondary to the electrical remodeling, even if LV geometry remains unchanged. VAT in precordial V6 readings gave the best matching results with the mean value of VAT in 12 leads that – for simplicity – may be considered in clinical practice (Boles et al. 2010).

The importance of the diagnosis of diastolic heart dysfunction in the community is linked to the wide prevalence of hypertension. Since hypertension is an asymptomatic and insidious disease, at the beginning, early ECG signs for electrical remodeling may provide a great deal of information in disease stratification.

Ironically, we can screen every patient with hypertension for diastolic heart dysfunction with echocardiography without the need to master the knowledge to criticize the ECG; however, the electrocardiogram remains widely accessible, robust, and cheap and requires less expertise to perform. On the other hand, whether applying the ECG as a cost-effective technology to screen for those groups of the population remains undetermined and requires further studies on a larger scale in a more diverse population to include all pathologies of diastolic dysfunction.

Summary of Points

- This chapter focuses on new electrocardiogram (ECG) markers in diastolic dysfunction secondary to hypertension.
- Diastolic dysfunction and hypertension are commonly diagnosed, and they have significant implications for health. Hence, using available diagnostic tools may provide earlier detection.
- Diastolic dysfunction may be associated with electrical remodeling without any evidence for myocardial structural changes, i.e., LVH.
- Ventricular activation time represents the conduction time of electrical depolarization and travel from compact AV node through the His bundle to the Purkinje fibers.
- This period is easy to interpret on the surface ECG as a QR interval, i.e., from the early deflection of QRS duration to the peak of the R wave.
- Atrial changes in diastolic dysfunction are common and are secondary to increasing atrial pressure from impaired ventricular relaxation and elevated left ventricular diastolic pressure.
- This can subsequently lead to atrial myocardial stretch and fibrosis and hence delay in atrial pulse propagation.
- Atrial changes are commonly seen with long P wave dispersion, i.e., the difference between the longest and shortest P wave durations on 12-lead ECG.
- Also, depicted in P wave terminal force in lead V1 (PTFV1) on the surface ECG. This represents the multiplication of the terminal P wave in V1, duration, and voltage. This was previously appreciated as a potential predictor of cardiac mortality and morbidity.
- PTFV1 of more than 40 mm.ms may also predict diastolic dysfunction and correlates with disease severity.
- In this chapter, we provide a critical review of new ECG markers in VAT, PWD, and PTFV1 in diastolic dysfunction highlighting electrical remodeling concepts that may occur before any detectable myocardial changes.
- The abovementioned ECG parameters are easy and effective tools for diagnosis and may direct early management of the condition by echocardiography and early treatment of the underlying pathology.

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Markers of Cardiac Resynchronization Therapy

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_51

Abstract

Despite the well-recognized benefits of CRT, an unsolved problem is the fact that based on the current selection criteria up to 30 % of patients do not respond to this therapy. Therefore, it is of paramount importance to try to identify more precisely patients who will derive the best benefit of this invasive therapy. Patient selection for CRT should involve a multimodal approach, and new promising tools may help in this difficult process. In this chapter, we will briefly discuss the impact of CRT in the expression of several biomarkers and also their role as predictors of CRT response, namely endothelial progenitor cells, brain natriuretic peptide, inflammatory mediators, biological markers, and renal function.

Keywords

Heart failure • Cardiac resynchronization therapy • Predictors of response • Inflammatory mediators • Endothelial progenitor cells • BNP • Renal function

Abbreviation

BNP	Brain natriuretic peptide
CKD	Chronic kidney disease
CRT	Cardiac resynchronization therapy
EPCs	Endothelial Progenitor Cells
GFR	glomerular Filtration Rate
HF	Heart Failure
hs-CRP	high sensitivity C Reactive Protein
ICTP	carboxyterminal telopeptide of type I collagen
LV	Left Ventricular
LVESV	left ventricular end-systolic volume
MDRD	Modification of Diet in Renal Disease
NYHA	New York Heart Association
NT- proBNP	terminal fragment pro-brain natriuretic peptide
NP	Natriuretic peptides
HF	Heart Failure
TNFα	Tumor Necrosis Factor α

Introduction

The normal functioning of the heart depends on the sequential activation of its components throughout the cardiac cycle, which requires the integrity of the electrical conduction system. The term *ventricular dyssynchrony* refers to the altered timing and pattern of ventricular contraction due to electrical disturbances or distorted electrochemical substrate, which might compromise the pumping capacity of the heart. These disorders are common in patients with heart failure, in particular when there are disturbances in the conduction system, such as bundle branch blocks.

The ventricular conduction delays produce suboptimal ventricular filling, reduction in left ventricular contractility, increased mitral regurgitation, and abnormal septal wall motion, thus affecting the performance of an already dysfunctional heart (Abraham 2015; Dickstein et al. 2010; Daubert et al. 2012; Brignole et al. 2013).

The electrocardiographic definition of ventricular dyssynchrony consists in an increased duration of the QRS complex (above 120 milliseconds) in the surface electrocardiogram, reflecting delayed ventricular activation. One-third of patients with systolic heart failure meet these criteria, and nowadays it is possible to treat this disturbance with pacing devices (cardiac resynchronization therapy – CRT). In brief, a pacing lead is implanted in the coronary sinus to pace the left ventricle, and another lead is placed in the right ventricle, thus improving the synchrony of ventricular activation. There is an increased stroke volume of the left ventricle after this therapy; the chronic benefits include left ventricle reverse remodeling with a reduction in left ventricular end-systolic and end-diastolic volumes, which is associated with an improvement in ejection fraction. Furthermore, tackling dyssynchrony significantly improves left ventricular mechanics with reduction of functional mitral regurgitation (Abraham 2015; Dickstein et al. 2010; Daubert et al. 2012; Brignole et al. 2013).

CRT has been studied in symptomatic patients with depressed ejection fraction and electrocardiographic criteria of ventricular dyssynchrony in several randomized controlled trials (MUSTIC, MIRACLE, MIRACLE ICD, CONTAK CD, CARE-HF, COMPANION, MADIT-CRT, REVERSE, and RAFT trials) (Linde et al. 2002; Abraham et al. 2002; Young et al. 2003; Achtelik et al. 2000; Cleland et al. 2005; Bristow et al. 2004; Moss et al. 2009; Linde et al. 2008; Tang et al. 2010). Overall, CRT improves symptoms and exercise tolerance, reduces heart failure hospitalization by 50 %, and diminishes mortality by 35 %. Based on these studies, CRT with biventricular pacing is recommended in symptomatic patients (NYHA functional class II, III, or IV) on optimal medical treatment with reduced left ventricular ejection fraction ($\leq$35 %) and prolonged QRS duration (above 120 milliseconds if left bundle branch block morphology, above 150 milliseconds if other morphologies) (Dickstein et al. 2010; Brignole et al. 2013).

Despite the formal recommendations and overall benefits of CRT, there are some unresolved issues. First, the implantation of both leads is technically feasible in 88–92 % of the procedures and carries a small risk of coronary sinus lesion, thus hindering some patients from its benefits. Secondly, around 30 % of the patients with biventricular pacing do not respond to this therapy (Dickstein et al. 2010; Brignole et al. 2013).

Several criteria have been proposed to define CRT response. Some entail clinical measures, such as symptomatic functional class improvement, reduced hospitalizations, and superior quality of life; these are subjective and prone to placebo effect. Echocardiographic criteria are more objective, namely increased ejection fraction and reduced left ventricular end-diastolic volume, the latter a marker of reverse remodeling. Considering the plethora of response criteria, up to 50 % of patients are classified as nonresponders. Since CRT is expensive and is not without hazard, it seems sensible to try to identify more precisely those who will derive the best benefit

and those least likely to, as in this latter group the cost-effective equation will be dramatically different (Yu and Hayes 2013; Yu et al. 2010).

There are subgroups of patients who show better response to CRT: female gender, those with wider QRS duration, left bundle branch block morphology, nonischemic heart failure etiology, and without significant scarred myocardium. Some authors have explored the role of several imaging techniques in predicting response to CRT, but the results have been disappointing. Therefore, it is of paramount importance to identify better predictors of CRT response (Yu CM, Hayes DL 2013; Yu CM et al. 2010).

Apart from the mechanical dyssynchrony effect of CRT, there is growing data on the "reverse cellular remodelling" following effective biventricular pacing. Some studies compared changes in cellular signaling pathways by CRT in responders versus "nonresponders," showing that myocardial gene expression changes of calcium handling proteins and natriuretic peptides were reversed preferentially in responders. Moreover, successful CRT is associated with decreased circulating biomarkers of extracellular matrix remodeling, such as tenascin-C and matrix metalloproteinase 9, and anti-inflammatory effects with reduced chemoattractant protein-1, interleukin-8, and interleukin-6 levels. Patients with effective CRT display chronic enhancement of circulating apelin, a secreted hormone that can block adverse remodeling and has positive inotropic effects (Cho et al. 2012).

The knowledge of the mechanisms involved in reverse cellular remodeling response has led to its application in CRT response prediction. For instance, studies using a metabolomic approach concluded that altered free fatty acid flux and calculated maximal adenosine triphosphate synthesis could be used to predict nonresponse to CRT, due to impaired energy efficiency. Likewise, several biomarkers are being studied in their abilities to predict CRT response (Barth et al. 2012).

In this chapter, we will explore the impact of CRT in the expression of several biomarkers and also their role as predictors of CRT response, namely endothelial progenitor cells, brain natriuretic peptide, inflammatory mediators, biological markers, and renal function.

Cardiac Resynchronization Therapy and BNP/NT-proBNP

Despite treatment with angiotensin-converting enzyme inhibitors, beta-blockers, and aldosterone antagonists, morbidity and mortality remains high in patients with chronic heart failure. The prognosis is even worse in patients with HF who have prolonged QRS intervals. This may reflect cardiac dyssynchrony and a greater propensity to adverse ventricular remodeling Fruhwald et al. 2007.

CRT with or without a defibrillator has been shown in several large randomized controlled trials to be effective at reducing symptoms, hospitalization time, and mortality in HF patients. However, despite its success in large studies, a lack

of response to CRT has been reported in up to one-third of device recipients Brenyon et al. 2013.

The issue of CRT "response" remains controversial. There is no good definition of a "responder" or "nonresponder." The fact that a patient's symptoms may not have improved, or the left ventricular volumes have not reduced, is used by many to indicate lack of response, but such an approach ignores the fact that patients may have had a mortality benefit, or might (without device) have deteriorated further. Approximately 70 % of patients who undergo CRT feel better. However, there is a large placebo response to CRT as demonstrated in MIRACLE group McDonagh et al. 2011.

Several factors, including high BNP levels, have been proposed as predictors of poor response to CRT.

Some patients respond spectacularly well to CRT and some deteriorate. A subanalysis of the PROSPECT study defined super-responders as having a reduction in left ventricular end-systolic volume (LVESV) of 30 % or more, responders a reduction of 15–29 %, nonresponders a reduction of 0–14 %, and "negative responders" an increase in LVESV. Super-responders were more frequently female, had nonischemic HF, a wider QRS complex, and more extensive dyssynchrony at baseline. The reported percentages of clinical responders and non- responders are shown in Fig. 1.

While it is important to identify patients who are most likely to respond to CRT, it is perhaps more important to identify patients in whom CRT may actually be harmful; in that way, BNP and NT-proBNP have been suggested to be a useful tool in both pre-CRT risk stratification and in monitoring for post-CRT response Brenyon et al. 2013.

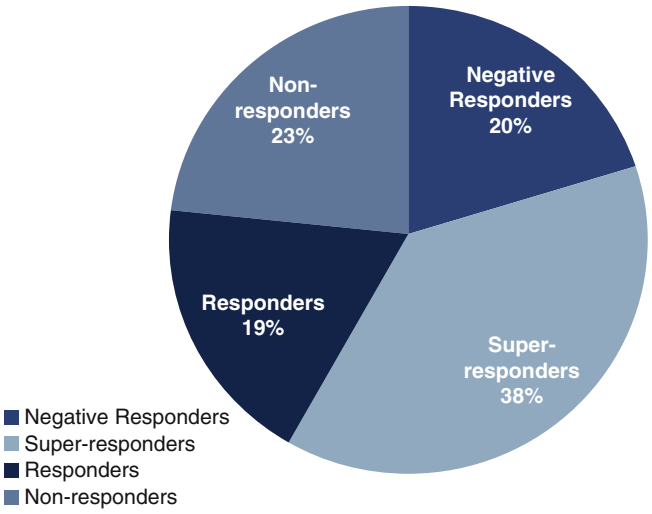

Fig. 1 Percentage of responders according to the extend of reduction in LVESV (Adapted of Oxford Textbook of Heart Failure 2011)

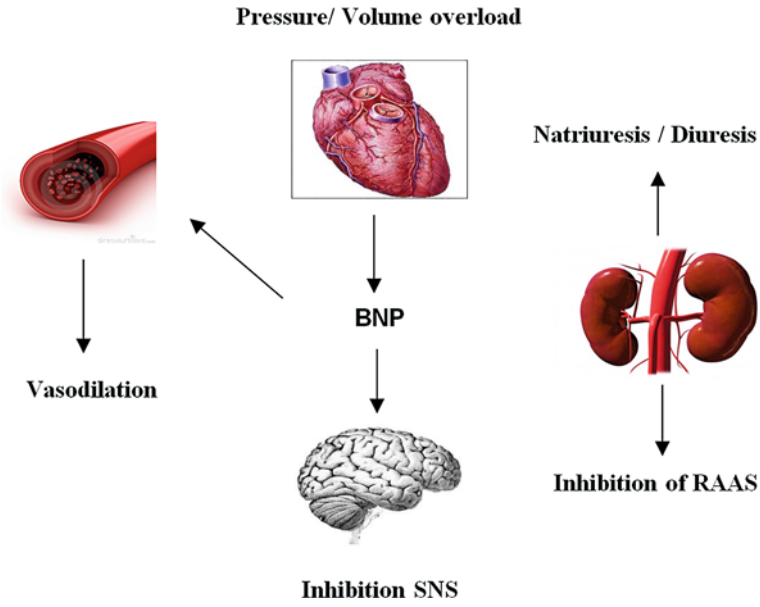

Fig. 2 Physiological effects of B- type natriuretic peptide (BNP). *RAAS* Renin–angiotensin–aldosterone system, *SNS* Sympathetic nervous system

Early and Sustained Effects of Cardiac Resynchronization Therapy on Biomarkers (BNP/NT- proBNP)

As indicated before, BNP and NT-proBNP are produced by ventricular cardiomyocytes in response to myocardial stretch and elevated ventricular filling pressures (Fig. 2). A higher baseline plasma concentration predicts a higher risk of all-cause mortality, sudden death, and death from heart failure. Elevated BNP at the time of CRT is prognostic of subsequent HF or death independently of the type of the device received. In some trials, CRT is associated with significant reductions in BNP levels during the follow-up time, whereas a similar pattern is not observed among patients who are not treated with the device Brenyon et al. 2013.

The CARE-HF trial demonstrates that CRT exerts a remarkable early and sustained reduction in plasma concentrations of NP levels when compared with pharmacological therapy alone in patients with moderate to severe chronic HF and ventricular dyssynchrony. These changes were most strongly associated to improvements in left ventricular function and reductions in mitral regurgitation. CRT has a more or less instantaneous effect on cardiac function and mitral valve regurgitation. The early reduction in NP shown in CARE-HF trial probably reflects the acute hemodynamic improvement that should reduce ventricular filling pressure and improve efficiency, and this would be expected to lead to beneficial ventricular remodeling (Berger et al. 2009).

The pattern of BNP change and the absolute BNP value at 1 year after CRT implantation is related to the echocardiographic response to the device and the risk of HF or death. Indeed, NT-proBNP may be the most robust, simple, objective prognostic marker in patients with HF. If the NPs are robust guides to prognosis, then it might be expected that change in NP might be a useful guide to the effectiveness of therapy Brenyon et al. 2013.

Plasma concentrations of NP might be used to guide changes in diuretic therapy, the need to increase doses of cardioprotective medication, and perhaps to guide when to implement CRT or implantable defibrillators. If natriuretic peptides are adopted as therapeutic target in patients with HF, then CRT appears to be a powerful additional intervention to achieve such a target in appropriately selected patients.

Conclusion

In an era in which the number of eligible candidates for CRT continues to increase, identifying optimal candidates for the therapy becomes especially important. In addition to device enhancements to individualize treatment and imaging modalities to detect ventricular dyssynchrony, monitoring BNP levels at baseline and during follow-up may be a powerful tool to further assess the response of patients with symptomatic HF treated with CRT.

Contribution of Inflammatory Mediators and Cardiac Extracellular Matrix Metabolism as Predictors of Response to Treatment by Cardiac Resynchronization Therapy

Heart failure (HF), the final common pathway for most cardiovascular conditions, incorporates a complex network of numerous molecular and cellular events that translates into profound alterations in structure and function of the cardiovascular system. The understanding of the complex pathophysiological mechanisms that underlie the syndrome of HF is constantly evolving, making the task of developing a single integrated theoretical model encompassing all aspects of this disease extremely challenging. Nevertheless, it is widely accepted that HF is triggered by an index event – an acute or chronic myocardial injury that impairs the pumping capacity of the heart. In order to counteract the impairment caused by the index event, autonomic, hormonal, immune, and inflammatory systems are activated with an initial protective role, trying to achieve a new level of homeostatic balance. However, continuous excessive activation of these initial compensatory mechanisms leads to detrimental consequences within the myocardium that are the base of progressive worsening HF and are referred collectively as cardiac remodeling (Gong et al. 2007). Therefore, cardiac remodeling can be defined as an adaptation of cellular and extracellular compartments of the heart to mechanical, hormonal, autonomic, and inflammatory stimuli that act in response to an index injury and lead

to detrimental modifications in structure and function of the heart (Rienks et al. 2014). In the pursuit of improving the prognosis of HF patients, current pharmacological and device therapies try to act on this deleterious remodeling process, aiming the reversion of the biological changes that constitutes the basis of the worsening cascade of HF.

A subset of patients with chronic HF present important abnormalities in ventricular conduction of electric stimuli that alter the timing and pattern of ventricular contraction, leading to suboptimal ventricular filling and contraction and prolonging the duration of mitral regurgitation. All these hemodynamic constraints impose an additional mechanical disadvantage to an already failing heart. CRT, through promotion of coordinated biventricular pacing, corrects this electromechanical dyssynchrony and eventually may induce a reverse cardiac remodeling, breaking the vicious cycle of heart failure progression. Much attention has been given to the molecular and cellular mechanisms that may underlie reverse cardiac remodeling induced by CRT. One of the most active lines of investigation focuses on the modulating effects of CRT on inflammatory mediators and cardiac extracellular metabolism. Besides shedding light into the complex network of HF pathogenesis, clarifying the molecular pathways underlying the reverse remodeling capability of CRT offers a huge translational opportunity to investigate potential predictors of CRT response in HF patients. In fact, in spite of its potential benefits, approximately 30 % of patients implanted with CRT devices do not show clinical improvement. CRT nonresponse remains a major clinical problem fueling an intense investigation in the pursuit of reliable predictors of CRT response in order to identify the so-called nonresponders before CRT implantation. Increasing the complexity of the subject, multiple definitions of CRT response have been proposed, namely a clinical response assessed by exercise capacity tests, quality-of-life questionnaires, and frequency of heart failure hospitalizations, heart transplantation, and cardiovascular death and an echocardiographic response assessed through change in left ventricular volumes, ejection fraction, or cardiac output (Brouwers et al. 2014).

The following section is on the role of inflammatory and extracellular matrix metabolism biomarkers in the assessment of response to cardiac resynchronizing therapy in heart failure patients and their potential application in improving patient selection to CRT.

Inflammatory Mediators

Persistent immune activation is a central feature in HF pathophysiology, comprehending a deregulated interplay of proinflammatory and inhibitory cytokines that exert toxic effects on both the heart and peripheral tissues. At the cellular and molecular level cytokines participate in the process of cardiac adverse remodeling by promoting myocyte hypertrophy, myocyte apoptosis, contractile dysfunction, and changes in the composition and structure of extracellular matrix Mann (2002). It has been shown that cytokines may be released from both the heart itself in response to end-diastolic wall stress and adrenergic activation and from peripheral tissues in

response to stagnant hypoxia and endotoxins released into circulation by translocated intestinal bacteria Rubaj et al. (2006).

The effect of CRT on inflammatory biomarkers has been inconclusive. Despite some contradictory reports published so far, probably a result of a marked heterogeneity in study design and a reduced number of patients, there seems to be emerging converging evidence in favor of a beneficial effect of CRT on inflammatory parameters. The most likely explanation for this is that corrected electromechanical dyssynchrony may have a potential to decrease local and peripheral production of inflammatory mediators McAlister et al. (2004). In fact, corrected electromechanical dyssynchrony may reduce the mechanical stress of the late-activated lateral wall of left ventricle leading to an improvement of global cardiac loading conditions and thus reducing the stimulus for local production of cytokines. On the other hand, improving electromechanical synchrony will improve cardiac output and consequently tissue perfusion reducing the inflammatory stimulus represented by local ischemia and possible intestinal bacterial translocation.

Selected studies regarding the prognostic role of inflammatory biomarkers on CRT outcome are resumed in Table 1. A multitude of inflammatory mediators have been studied, but most evidence regards high-sensitivity C Reactive Protein (hsCRP) and Tumor Necrosis Factor α (TNFα).

High-sensitivity C Reactive Protein: hsCRP is synthesized and secreted by hepatocytes in response to proinflammatory cytokines and contributes to HF progression by upregulating the production of macrophage proinflammatory cytokines (IL-6, TNF-α, and IL1β), oxygen species formation, and expression of enzymes responsible for extracellular matrix turnover. In terms of HF prognosis, hsCRP has solid evidence pointing to association of high levels with increased mortality Rubaj et al. (2013).

Regarding the prognostic impact of hsCRP levels on CRT response, evidence has been conflicting. Brouwers et al., Theodarakis et al., and Glick et al. did not find significant differences in either baseline or after CRT implantation hsCRP levels between CRT responders and nonresponders Glick et al. (2006); Theodorakis et al. (2006); Brouwers et al. (2004). However, Cai et al., Rujab et al., and Kamioka et al. found that CRT responders had lower baseline and a greater decrease in hsCRP levels than nonresponders Rubaj et al. (2006); Kamioka et al. (2012); Cai et al. (2014) .

Tumor Necrosis Factor α: TNFα has been implicated in several aspects of HF pathogenesis by exerting a negative inotropic effect, triggering apoptosis in cardiomyocytes, and activating enzymes that degrade extracellular matrix Rordorf et al. (2014). In combination with IL6, TNFα and its soluble receptors are stronger predictors of HF mortality than traditional factors such as NYHA class, left ventricular ejection fraction, and maximal oxygen consumption Rauchhaus et al. (2000). Recently has emerged the concept that TNFα may be able to provide information on the degree of remodeling in patients with HF, with higher levels being associated to more advanced and possibly irreversible remodeling Rordorf et al. (2014). Similar to hsCRP, evidence regarding the prognostic impact of TNFα levels on CRT response is not consensual. On the one hand, Rordorf et al. (2014). found that the rate of

Table 1 Selected studies concerning inflammatory mediators and their prediction of cardiac resynchronization therapy response

Author (year)	Study design	Number of patients	Biological markers	Follow-up (months)	Assessment of CRT response	Main results
(Stanciu et al. 2013)	Prospective study Repeated measures design (before and after CRT implantation)	27	IL-1β, IL-6 and IL-8	12 months	CRT responder defined as more than 10 % reduction in left ventricle end systolic volume and improvement in NYHA functional class in at least one class and no heart transplantation or death caused by cardiovascular causes	Circulating levels of IL-1β, IL-6 and IL-8 decreased from their baseline values
(Boriani et al. 2006)	Prospective study Repeated measures design (before and after CRT implantation)	32	IL-6, TNF and soluble TNF receptors 1 and 2 (sTNFR1 and sTNFR2)	3 months	CRT responder defined as ≥1 NYHA class reduction or ≥15 % reduction in left ventricle end systolic volume	Serum levels of IL-6, TNF and sTNFR1 and sTNFR2 did not change from baseline to follow up Baseline levels of IL-6, TNF and sTNFR1 did not differ between CRT responders and non responders
(Brouwers et al. 2014)	Prospective study Repeated measures design (before and after CRT implantation)	105	CRP, IL-6, TNFα, sTNFR1 and sTNFR2	14 months	Objective CRT response defined as a reduction of ≥15 % in left ventricular end systolic volume and Subjective CRT response defined as an improvement of ≥10 points in patient-reported health status assessed with the Kansas City Cardiomyopathy Questionnaire	Baseline concentrations of inflammatory markers did not differ between the objective CRT responders and non responders. Both objective CRT responders and non responders showed a significant decrease in TNFα levels from baseline to 14 months follow up. The other inflammatory markers did not change significantly over time in both groups The subjective CRT responders had significantly lower baseline levels of TnFα compared to non responders. Subjective responders showed a significant decrease in BNP, TNFα and sTNFR1 levels from baseline to 14 months follow

						up. Subjective non responders had a decrease in TNFα over time and an increase in IL-6, sTNFR1 and sTNFR2 levels
(Cai et al. 2014)	Prospective study Repeated measures design (before and after CRT implantation)	232	High sensitivity C-reactive protein (hsCRP)	6 months	CRT responders defined as ≥1 NYHA class reduction combined with an absolute increase ≥5 % in LVEF during 6 month follow up	Baseline hsCRP values as well as 6-month hsCRP levels were significantly lower in patients who responded to CRT than in those who did not Patients with low 6 month hsCRP values displayed the largest reduction in LVEDD and increases in LVEF
(Glick et al. 2006)	Prospective study Repeated measures design (before and after CRT implantation)	32	hsCRP	2 weeks	CRT responder defined as ≥1 NYHA class reduction after 1 month	Levels of hsCRP decreased significantly 2 weeks after implantation. Baseline levels of hsCRP or their drop after 2 weeks of CRT implantation did not correlate with clinical outcome
(Kamioka et al. 2012)	Prospective study Repeated measures design (before and after CRT implantation)	65	hsCRP	6 months	CRT responder defined as >15 % reduction in LVESV	Baseline hsCRP was significantly higher in patients who did not respond to CRT than in those who did HsCRP was the strongest predictive factor for cardiac death
(Lappegard and Bjornstad 2006)	Prospective study Repeated measures design (before and after CRT implantation)	8	Monocyte chemoattractant protein 1 (MCP-1), interleukin-8 (IL-8), interleukin 6 (IL-6), interleukin 1β (IL-1β), tumor necrosis factor α (TNFα), interleukin 10 (IL-10) and complement activation products	6 months	Not defined	There was a significant reduction in plasma levels of MCP-1, IL-8 and IL-6. No changes were observed in the levels of IL1β, TNFα, IL-10 and complement activation products
(Michelucci et al. 2007)	Prospective study Repeated measures design (before and after CRT implantation)	140	IL-6, hsCRP	6 months	Not defined	In patients who survived free from clinical events, IL-6 and hsCRP levels significantly decreased from baseline to 6 months follow up. In patients who has an

(*continued*)

Table 1 (continued)

Author (year)	Study design	Number of patients	Biological markers	Follow-up (months)	Assessment of CRT response	Main results
						adverse clinical event, no significant changes were observed in both IL-6 and hsPCR from baseline to 6 months follow up
(Przybyla et al. 2011)	Prospective study Repeated measures design (before and after CRT implantation)	38	IL-6, interleukin 18 (IL-18), CRP	3 months	Not defined	The level of IL-6 decreased from baseline to 3 months follow up. No change in CRP and IL-18 levels were observed
(Rordorf et al. 2014)	Prospective study Repeated measures design (before and after CRT implantation)	91	TNFα and IL-6	6 months	CRT response defined as a decrease ≥15 % in left ventricular end systolic volume at 6 months	Baseline circulating IL-6 was not correlate with reverse remodeling. Baseline circulating IL-6 did not predict response to CRT Baseline TNFα was significantly predictive of left ventricular end systolic volume reduction. The rate of response to CRT was significantly different according to baseline circulating TNFα: there was a linearly decreasing proportion of patients with LVESV reduction ≥15 % from the lower through the intermediate to the upper tertile of TNFα level Patients with the higher baseline circulating TNFα had the worst clinical outcome (composite endpoint of cardiac death, heart failure hospitalization and urgent cardiac transplantation)

(Rubaj et al. 2006)	Prospective study Repeated measures design (Rignt ventricular pacing to biventricular pacing)	28	CRP, TNFα and IL-6	48 h	CRT responder defined as an increase >10 % in cardiac output	TNFα and IL6 measured during RV pacing (baseline) did not differ between the responders and the non responders Levels of TNFα and IL-6 significantly decreased following the change in pacing mode from right ventricular pacing to biventricular pacing Levels of CRP did not change following the change in pacing mode
(Rubaj et al. 2013)	Prospective study Repeated measures design (Biventricular pacing followed by Rignt ventricular pacing followed again by biventricular pacing)	54	High sensitivity CRP, IL-6	48 h after CRT-off	CRT responder defined as improvement of at least 1 NYHA class and absolute increase in LVEF >10 %	A significant increase in serum concentrations of hsCRp and IL-6 was observed in CRT responders after CRT interruption. When CRT was switched on again, a significant reduction in IL-6 and cRP was observed in this subgroup of patients. In CRT non responders, switching biventricular pacing to right ventricular pacing did not lead to significant changes in hsCRP and IL-6
(Shinohara et al. 2011)	Prospective study Repeated measures design (before and after CRT implantation)	27	Hs-CRP	6 months	CRT responder defined as ≥15 % absolute decrease in left ventricular end systolic volume	In responders, serum levels of hs-CRP significantly decrease after 6 months follow up. Non responders did not show any change in hs-CRP levels after 6 months follow up
(Tarquini et al. 2009)	Prospective study Repeated measures design (before and after CRT implantation)	47	TNFα, TNF soluble receptors sTNFR1 and sTNFR2	12 months	CRT responder defined as ≥15 % absolute decrease in left ventricular end systolic volume	Baseline levels of TNFα and sTNFR1 and sTNFR2 were not significantly different in responders and non-responders No significant changes in levels of TNFα and sTNFR1 and sTNFR2 were observed after 12 months of CRT

(*continued*)

Table 1 (continued)

Author (year)	Study design	Number of patients	Biological markers	Follow-up (months)	Assessment of CRT response	Main results
(Theodorakis et al. 2006)	Prospective study Repeated measures design (3 months of no pacing followed by 3 months of biventricular pacing followed by 3 months of no pacing again)	20	CRP, TNFα, sTNFR1 and sTNFR2, adhesion molecules sICAM-1 and sVCAM-1	3 months + 3 months	Not defined	TNFα and its receptors showed a decrease after 3 months of pacing and a further decrease after the ensuing 3 months of no pacing. IL-6 and sICAM-1 levels decreased after 3 months of biventricular pacing and remained lower during the 3 months off pacing. No changes were observed in the levels of CRP and sVCAM-1
(Marin et al. 2011)	Prospective study No control group	36	CRP	4 months	Not defined	Following CRT, CRP levels remained unchanged

response to CRT was significantly different according to baseline TNFα – from the lower to the upper tertile of TNF-α, left ventricular volumes were progressively reduced after CRT Rordorf et al. (2014). On the other hand, Tarquini et al. showed that baseline levels of TNFα were not significantly different in CRT responders and nonresponders Tarquini et al. (2009).

Cardiac Extracellular Matrix Metabolism

The cardiac extracellular matrix is a metabolic active network consisting of proteins in which cardiac cells reside. Besides its plastic role, conferring support to efficient contraction and relaxation of cardiomyocytes, the cardiac matrix plays an important role in mediating cellular crosstalk and metabolic exchange (Li et al. (2014)).

Adverse cardiac remodeling during the course of HF is accompanied by changes in the structure and composition of extracellular matrix. It has been proposed that during early stages of HF inflammation favors collagen degradation that contributes to ventricular dilatation. As heart failure evolves, inflammation becomes chronic and different molecular pathways are activated with the resultant event being excessive collagen deposition instead of collagen degradation Mann (2002). The result of this metabolic shift is the development of undue myocardial stiffness that impairs both filling and pumping functions and provides a structural subtract to arrhythmogenicity.

Debate still exists regarding the potential effects of CRT on extracellular matrix metabolism. Nevertheless, most evidence points to a beneficial effect of CRT, which counteracts the persistent fibrogenesis of advanced HF and hence promotes reverse remodeling. Recently, some extracellular matrix biomarkers have emerged as useful tools in the prediction of CRT response as it is believed that subsets of HF patients in different metabolic stages of extracellular matrix remodeling may derive disproportionate benefit from this therapy. Most evidence regarding this subject concerns enzymes involved in collagen metabolism and galectin-3.

Collagen: Collagen type I and collagen type III are the main proteins of cardiac extracellular matrix. While collagen type I with its thicker fibers provides tensile strength to extracellular matrix, collagen type III being thinner yields elasticity. Both types of collagen are synthesized by fibroblasts from the assembly of three procollagen- α- chains. During collagen synthesis, amino and carboxy propetides of procollagen I and III (PINP, PICP, PIIINP, PIIICP) are cleaved and released into circulation. Collagen fibers are degraded by enzymes called metalloproteinases (MMPs) that can be inhibited by specific tissue inhibitors of metalloproteinases (TIMPs). As a result of MMP action during collagen degradation, small peptides may be released into the circulation as it is the case of the carboxyterminal telopeptide of type I collagen (ICTP). Collagen metabolism can be easily assessed noninvasively by measuring the ratio of MMP to TIMP activity or the levels of collagen's circulating by-products – PINP, PICP, PIIINP, and PIIICP to evaluate collagen type I and type III synthesis, respectively, and ICTP to evaluate collagen type I degradation. Regarding the prognostic impact of collagen metabolism on CRT

Table 2 Selected studies concerning cardiac extracellular matrix metabolism biomarkers and their prediction of cardiac resynchronization therapy response

Author (year)	Study design	Number of patients	Biological markers	Follow-up (months)	Assessment of CRT response	Main results
(Dong et al. 2011)	Prospective study Repeated measures design (before and after CRT implantation)	45	Amino-terminal propeptide of type III procollagen (PIIINP)	3 and 6 months	CRT responder defined as 15 % or greater reduction in left ventricular end-systolic volume index	The baseline PIIINP was lower in CRT responders than non responders PIIINP remained unchanged after 6 months of CRT
(Francia et al. 2011)	Prospective study Repeated measures design (before and after CRT implantation)	18	Osteopontin (OPN) –TGFβ1 axis	8,5 + - 4 months	CRT responder defined as 10 % or greater reduction in left ventricular end systolic volume	CRT responders showed a non significant trend towards higher baseline plasma OPN and TGFβ1 as compared to non responders Compared to baseline, circulating levels of OPN were significantly reduced in CRT responders and increased in non responders. TGFβ1 showed a trend towards reduction in responders while unchanged in non-responders

(Garcia-Bolao et al. 2006)	Prospective study Repeated measures design (before and after CRT implantation)	38	Carboxy-terminal propeptide of type I procollagen (PICP)	12 months	CRT responder defined as increase in the distance walked in 6 min by more than 10 % and no heart transplantation or death caused by heart failure	Baseline PICP was higher in responders than non responders At 1 year PICP decreased in responders and increased in non responders
(Hessel et al. 2007)	Prospective study Repeated measures design (before and after CRT implantation)	64	Tenascin-C (TNC), matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9)	6 months	CRT responder defined as 10 % or greater reduction in left ventricular end systolic volume	At baseline no significant differences in TNC, MMP-2 and MMP-9 were observed between responders and non-responders At 6 months follow up, TNC and MMP-9 showed a significant reduction in responders while remained unchanged in non-responders MMP-2 levels remained unchanged in both responders and non-responders

(*continued*)

Table 2 (continued)

Author (year)	Study design	Number of patients	Biological markers	Follow-up (months)	Assessment of CRT response	Main results
(Li et al. 2013)	Prospective study Repeated measures design (before and after CRT implantation)	27	MMP-9, tissue inhibitor of MMP-9 (TIMP-1), carboxyterminal propeptide of type I procollagen (PICP) and carboxyterminal telopeptide of type I collagen (ICTP)	3 months	Not defined	At 3 months follow up, the CRT group showed a significant reduction in MMP-9, TIMP-1, ICTP and MMP9/TIMP-1 and a significant increase in the PICP/ICTP ratio levels compared with pretreatment values At 3 months follow up, there was a negative correlation between left ventricle end diastolic diameter indexed to body surface area and the PICP/ICTP ratio in the CRT group

(Lopez-Andres et al. 2012)	Prospective study based on a previous randomized controlled trial (CRT vs no CRT)	260	Galectin-3 (Gal-3), PIIINP, amino-terminal popeptide of type I procollagen (PINP), ICTP and matrix metalloproteinase-1 (MMP-1)	18 months	Treatment efficacy assessed according to three criteria from the CARE-HF trial: survival without unplanned hospitalization for worsening heart failure; survival and left ventricle ejection fraction >35 %; survival and NT-proBNP levels <1000 pg/mL	There was no difference in median baseline levels of extracellular cardiac matrix biomarkers between control and CRT groups and mean values varied little throughout the 18 month follow up in both treatment groups Extracelullar cardiac matrix biomarkers at baseline did not predict the response to CRT MMP-1 concentration was associated with the outcome of death or LVEF $\leq$ 35 % at 18 months
(Marin et al. 2011)	Prospective study Repeated measures design (before and after CRT implantation)	36	MMP-1, MMP-2, TIMP-1 and CITP	4 months	Not defined	Following CRT, MMP-1 and MMP-2 levels were significantly increased at 4 months TIMP-1 and CITP remained unchanged

(*continued*)

Table 2 (continued)

Author (year)	Study design	Number of patients	Biological markers	Follow-up (months)	Assessment of CRT response	Main results
(Stanciu et al. 2013)	Prospective study Repeated measures design (before and after CRT implantation)	27	MMP-2 and tissue inhibitor of matrix metalloproteinase-2 (TIMP-2)	12 months	CRT responder defined as more than 10 % reduction in left ventricle end systolic volume and improvement in NYHA functional class in at least one class and no heart transplantation or death caused by cardiovascular causes	Serum levels of MMP-2 and MMP-2/TIMP-2 ratio decreased from baseline during follow up LVEF correlated negatively with MMP-2/ TIMP-2 ratio at follow –up
(Stolen et al. 2014)	Prospective study based on a previous randomized controlled trial (CRT-D vs ICD-only)	654	Gal-3	12 months	Composite primary endpoint from MADIT-CRT: nonfatal heart failure event or death from any cause whichever occurred first	Patients with Gal-3 values in the highest quartile derived a disproportionately larger benefit from CRT-D in comparison with patients with ICD-only. The absolute reduction in primary event rate attributable to CRT-D relative to ICD-only in the high gal-3 group was 11,2 fewer primary events per 100 patients-year and in the low gal-3 group it was 2,1 fewer primary events per 100 patient-years

(Umar et al. 2008)	Prospective study Repeated measures design (before and after CRT implantation)	64	PINP, PIIINP, ICTP, TIMP-1 and pro MMP1	6 months	CRT responder defined as more than 10 % reduction in left ventricle end systolic volume	Responders demonstrated an increase in serum PINP and PIIINP during follow up. In non responders, serum PINP and PIIINP remained unchanged during follow up At baseline responders had significantly lower serum PINP than non responders Serum levels of ICTP, proMMP1, TIMP-1 and proMMP1/TIMP1 did not differ between baseline and follow-up nor did they differ between responders and non-responders
(Vondrakova et al. 2012)	Prospective study Repeated measures design (before and after CRT implantation)	18	Gal-3	2 months	Not defined	Serum levels of Gal-3 decreased from baseline to follow-up

response, evidence has been conflicting. Garcia-Bolao et al. found that baseline PICP was higher in responders than in nonresponders. On the other hand Umar et al. showed that responders had lower baseline PINP than nonresponders. Other inconsistencies concerning MMP/TIMP ratios and MMP levels have been reported.

Galectin-3: Galectin-3 (Gal-3) is a protein secreted by activated macrophages that plays an important role in promoting fibrosis through fibroblast proliferation and collagen synthesis. A prospective study derived from a randomized control trial has shown that patients with gal-3 levels in the highest quartile derived a disproportionately larger benefit from CRT-D in comparison with patients with ICD only.

Conclusion

CRT has assumed a central role in the treatment of HF patients with evidence of electromechanical dyssynchrony. However, at least 30 % of patients with implanted CRT devices do not show the expected clinical improvement. As we advance in the understanding of the cellular and molecular mechanisms that underlie the reverse remodeling promoted by CRT, novel biomarkers with the ability to accurately predict response versus nonresponse to CRT are expected to arise. Such a breakthrough with the consequent improvement in the selection of patients to CRT would entail a huge clinical and economic impact. In the pursuit of this objective, larger prospective studies with adequate design and longer follow-up times are needed.

Potential Applications of Circulating Endothelial Progenitor Cells in CRT

Currently, cardiac resynchronization therapy (CRT) using biventricular pacing is a standard of care in the management of advanced heart failure (HF) Brignole et al. (2013). However, based on current selection criteria, a considerable proportion of eligible patients still fail to benefit from this treatment Daubert et al. (2012). Identifying reliable predictors of effectiveness of CRT remains a major challenge in clinical practice, particularly from the perspective of patient selection.

Endothelial dysfunction is an important underlying mechanism in the pathophysiology of HF, which has recently been suggested as an independent predictor of CRT response Akar et al. (2008). Endothelial progenitor cells (EPCs) harbor a recognized capacity to proliferate and differentiate into mature endothelial cells, contributing in vivo to both reendothelialization and neoangiogenesis, and therefore to the maintenance of endothelial integrity Liao et al. (2010). Furthermore, it has been recently suggested that patients with higher circulating EPC levels have a greater neovascularization potential and are more likely to exhibit a positive response to CRT António et al. (2014).

Cardiac Remodeling in HF Patients and Reverse Remodeling After CRT

A common aspect of HF, irrespective of the underlying etiology, is the development of cardiac remodeling, which describes the changes in LV mass, volume, shape, and composition of the ventricle in response to the mechanical (stress and strain) and systemic neurohormonal activation. The alterations that occur in the failing myocardium may be divided into those that occur in the cardiac myocytes as well as those which occur in the extracellular matrix (Table 3). Ultimately, these changes lead to progressive LV dilation, increased sphericity of the ventricle, and progressive decline in contractile function Mann et al. (2012) and Li et al. (2014).

From several large clinical trials it is becoming increasingly clear that CRT leads to decreased left ventricular (LV) volume and mass, and restores a more normal elliptical shape of the ventricle. These salutary changes have been called "reverse remodeling" Linde et al. (2002), Abraham et al. (2002), Cleland et al. (2005), Moss et al. (2009). Remarkably, there are subsets of patients who undergo a reverse remodeling and whose clinical course is free of future heart failure events – myocardial recovery. However, exactly what causes this cardiac reverse remodeling resulting from CRT and what subcellular mechanisms are involved are only poorly understood. It is even less clear why a significant number of patients do not respond positively to CRT and why some patients exhibit molecular reverse remodeling but this does not translate to clinical recovery.

Endothelial Progenitor Cells as a Predictor of CRT Response

A growing body of evidence strongly demonstrates that endothelial dysfunction plays an important role in the pathogenesis and progression of HF. Moreover,

Table 3 Cellular, molecular and anatomic changes that occur during cardiac remodelling in HF patients

Myocyte defects	Myocardial defects		Reversal of abnormal LV geometry
Hypertrophy	Myocyte death	Apoptosis	LV dilation
Fetal gene expression		Necrosis	LV wall thinning
β-adrenergic desensitization		Autophagy	Mitral valve incompetence
Myocytolysis	Alterations in extracellular matrix	Matrix degradation	
Excitation contraction coupling		Replacement fibrosis	
Cytoskeletal proteins		Angiogenesis	
Myocyte energetics			

endothelial dysfunction seems to be correlated with disease severity and prognosis in HF patients. Of note, it has been recently demonstrated that endothelial function independently predicts CRT response Akar et al. (2008).

Endothelial progenitor cells are endothelial and hematopoietic progenitor cells having a recognized capacity to proliferate and differentiate into mature endothelial cells, contributing to the process of vasculogenesis, repairing the damaged and dysfunctional endothelium. As circulating EPC numbers seem to be related to endothelial function, EPCs have been proposed by Liao YF et al. as a surrogate biological marker of endothelial function Liao et al. (2010).

It has been demonstrated that circulating EPCs correlate with favorable left ventricular remodeling after myocardial infarction. Therefore, it is conceivable that circulating EPC levels also contribute for the reverse remodeling associated with CRT and influence the response to this therapy. Remarkably, we have published data showing a positive correlation between baseline EPC levels and LVESV reduction after CRT suggesting a role of EPCs in the reverse remodeling observed with resynchronization (Fig. 3). Additionally, in our work responders to CRT showed significantly higher levels of EPCs by comparison with nonresponders, reinforcing the hypothesis that EPCs may have an important role in reverse remodeling and CRT response António et al. (2014).

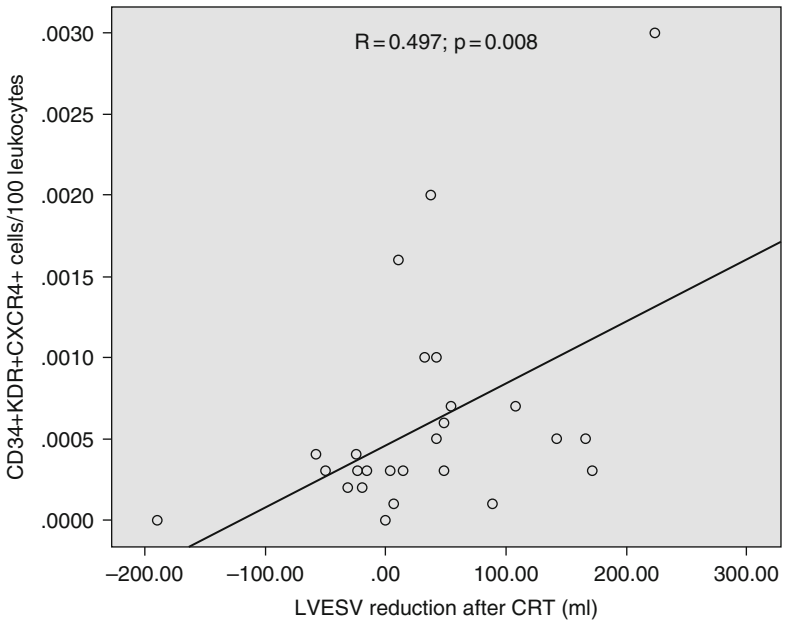

Fig. 3 Comparison of baseline EPCs levels between responders and non-responders to CRT (Adapted from Antonio N et al. Pacing Clin Electrophysiol. 2014)

Conclusions

Despite the high effectiveness of CRT in severe chronic HF, the rate of nonresponders remains an important problem. In fact, up to 30 % of patients treated with CRT do not exhibit the desirable reverse remodeling and cardiac recovery. Circulating EPCs, a surrogate marker of endothelial function, may help identifying the subset of HF patients with greater neovascularization potential and higher probability to undergo reverse remodeling and benefit from CRT. Therefore, the quantification of circulating EPC levels may be an important additional tool to identify the best CRT candidates.

Renal Function and Cardiac Resynchronization Therapy

Cardiac and renal functions have a well-known interdependent relationship as there are a number of important bidirectional interactions between heart and kidney diseases. Chronic kidney disease (CKD) is present in more than half of patients with heart failure (HF) and approximately two-thirds of patients hospitalized with HF have renal insufficiency (Smith et al. 2006; McAlister et al. 2004a). In both the acute setting and long-term phase of HF, even small decreases in glomerular filtration rate (GFR) are associated with an adverse prognostic impact (de Silva et al. 2006; Coca et al. 2007). CRT significantly improves outcomes in a group of patients with advanced HF and renal function can be considered to improve the selection of patients, having important prognostic implications.

The serum creatinine level is usually used as a surrogate to estimate GFR, as kidney function is related directly to the urine creatinine excretion and inversely to the serum creatinine. As serum creatinine is also affected by factors unrelated to renal function, such as age, sex, race, and lean muscle mass, two formulas are used widely to estimate kidney function from serum creatinine: Cockcroft-Gault and four-variable Modification of Diet in Renal Disease (MDRD).

While CRT represents one of the most important advances for the treatment of advanced HF, nonresponse in a large number of patients continues to be problematic. The renal function biomarkers have been studied in order to improve patient selection to CRT. In a subgroup analysis of CARE-HF trial, the benefit of CRT-P on global mortality and cardiovascular hospitalization was preserved in patients with GFR less than 60.3 mL/min/1.73 m^2 (Cleland et al. 2006). In the REVERSE trial, there was no evidence of differential reduction in the primary endpoint of clinical response considering the GFR, but patients with a GFR < 60 were observed to have less left ventricular structural remodeling (Linde et al. 2008; Mathew et al. 2012) . In an observational analysis of Adelstein et al. comparing outcomes of CRT-D patients with a cohort of similar patients who received an ICD only, patients with moderate CKD (GFR 30–59 mL/min/1.73 m^2) had a significant survival advantage with CRT, associated with improved renal and cardiac function (Adelstein et al. 2010). On the other hand, patients with baseline severe CKD (GFR <30 mL/min/1.73 m^2) had a

poor survival despite CRT-D, which appeared to confer little echocardiographic benefit despite modest improvement in renal function.

Although serum creatinine–based estimating equations to GFR have been the most studied, the role of other renal biomarkers on CRT management was already studied. The effect of the ratio of blood urea nitrogen (BUN) to creatinine on response to CRT therapy was considered in a post hoc subgroup analysis of the MADIT-CRT trial (Goldenberg et al. 2010). The patients were dichotomized into two groups using the BUN/creatinine ratio value of 18 and it was found that the reduction of HF hospitalization or death was greater in patients with higher ratio. The authors concluded that prerenal azotemia, reflected in high BUN/creatinine ratio, is a marker for decreased circulation blood volume and identifies patients at higher risk for HF and, hence, a group with better response to CRT.

Cystatin C is a cysteine protease inhibitor that is produced at a relatively constant rate from all nucleated cells, and serum cystatin C has been proposed to be a more sensitive marker of early GFR decline than plasma creatinine. A prospective study from Yamamoto et al. showed that serum cystatin C level prior to CRT device implantation independently predicts mortality and morbidity (Yamamoto et al. 2013). The association of cystatin C with mortality is even superior to that of serum BNP level, providing an accurate risk stratification of CRT patients.

In summary, despite the higher mortality associated in CKD patients, the benefit of CRT on clinical outcomes seems to be preserved. Renal biomarkers have been studied in this context and could identify subgroups of patients with better response rates to CRT.

Summary Points

- Despite the high effectiveness of CRT in chronic HF, a significant proportion of patients selected using conventional criteria do not appear to benefit from CRT.
- Identifying reliable predictors of effectiveness of CRT remains a major challenge in clinical practice.
- In order to reduce the percentage of nonresponders to CRT, it could be helpful to use new promising tools, such as inflammatory biomarkers, BNP, and endothelial progenitor cells, in a multimodal approach to improve patient selection.
- Monitoring BNP levels at baseline and during follow-up may be a powerful tool to further assess the response of patients with symptomatic HF treated with CRT.
- Circulating EPCs, a surrogate marker of endothelial function, may help identifying the subset of HF patients with greater neovascularization potential and higher probability to undergo reverse remodeling and respond to CRT.
- As we advance in the understanding of the cellular and molecular mechanisms that underlie the reverse remodeling promoted by CRT, novel biomarkers with the ability to accurately predict response versus nonresponse to CRT are expected to arise.

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Adhesive Properties of Neutrophils as a Possible Biomarker of Vascular Disease

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Contents

Abstract

The migration of leukocytes from blood vessels into inflamed tissues is an essential immunity component. Neutrophils are the first leucocytes to arrive at sites of infection or tissue injuries where they exhibit numerous effector functions. Neutrophil recruitment to inflamed vascular endothelium has been described as a multistep process modulated by chemokines, selectins, and integrins that engage in a stepwise manner to initiate intracellular signals and

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_24

adhesive bond formation. Thus, chemoattractant-triggered inside-out and integrin-initiated outside-in signaling events cooperate concurrently to increase integrin affinity to its ligands and to stabilize and prolong the arrest of circulating neutrophils. This process enables neutrophils to efficiently navigate the journey from the blood stream to inflammatory sites, which is critical for host defense. However, excessive recruitment of activated neutrophils has been observed to sometimes cause local tissue damage and contribute to the development of inflammatory disorders. Therefore, neutrophils have been implicated in the pathogenesis of both acute and chronic vascular inflammatory diseases. Vascular diseases represent major health problems worldwide. Therefore, due to the economic, social, and health impact, early and precise detections of new biomarkers are crucial to identify the exposed population. Accordingly, the present chapter summarizes recent findings in this area. The aims of this review are to focus on new insights of mechanisms that mediate neutrophil transmigration and to evaluate the adhesive properties of neutrophils as potential biomarkers for vascular diseases.

Keywords

Adhesive properties • Migration • Integrins • Neutrophils • Biomarkers • Methods and vascular diseases

Abbreviations

AMI	Acute myocardial infarction
CRP	C-reactive protein
CRP	C-reactive protein
DVT	Deep venous thrombosis
END	Neurological deterioration
ICAM-1	Intercellular Adhesion Molecule 1
IL-6	Interleukin-6
IL-8	Interleukin-8
LFA-1	Lymphocyte Function-associated Antigen-1 alphaL beta2 integrin
Mac-1	Macrophage antigen-1 alphaM integrin
PAR	Protease-activated receptors
PE	Pulmonary embolism
PSGL-1	P-selectin glycoprotein ligand-1
RVO	Residual vein occlusion
SCD	Sickle cell disease
TF	Tissue factor
TNF-α	Tumor necrosis factor- alpha
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very Late Antigen-4 alpha4 beta1 integrin
VTE	Venous thromboembolism
WBC	White blood cell

Key Facts of Adhesive Properties of Neutrophils

- Neutrophils comprise of 40–60 % of the leukocyte population in human blood and play a crucial role in defending the organism, digesting microorganisms by phagocytosis.
- In order to play the defense function in the organism, neutrophils must first receive the information of an existent infection and then migrate to the area infected through the endothelium line.
- Endothelium is a tissue that recovers the vascular beds internally. Besides coating and delimitation functions, it acts as a semipermeable membrane, regulating the molecules' traffic, controlling the regulation of blood flow in vascular resistance, and modulating the immune and inflammatory responses.
- Cytokines are proteins produced by various cells that send many stimulatory, modulatory, or inhibitory signals for the various cells of the immune system.
- Selectins are proteins present in endothelial and immune cells. They are responsible for the rolling of leukocytes over the vascular endothelium line. This is the beginning of a cascade of events that leads to the extravasation of neutrophils at sites of injury and to the inflammatory process.
- The interaction of selectins with their ligands results in a dramatic decline in the speed rate of neutrophils rolling, which allows the activation of proteins known as integrins.
- Integrins are also transmembrane proteins that promote the firm adhesion of neutrophils to the endothelium.

Definitions

Acute ischemic stroke Occurs when the blood supply to a part of the brain is cut off due to atherosclerosis or to a blood clot which has blocked a blood vessel.

Acute myocardial infarction Necrosis of myocardial tissue due to ischemia, usually due to the blockage of a coronary artery by a thrombus Acute ischemic stroke.

Biomarkers Measurable indicators of a certain biological state or condition.

Cytokines Cytokines are proteins secreted by cells which have a specific effect on the interactions and communications between cells to coordinate appropriate immune responses.

Deep vein thrombosis Formation of a blood clot (thrombus) within a deep vein.

"Inside-out" signaling A process in which stimuli received by cell surface receptors for chemokines, cytokines, and foreign antigens initiate intracellular signals that

impinge on integrin cytoplasmic domains and alter adhesiveness for extracellular ligands.

Integrins Transmembrane receptors responsible for cell-cell and cell-extracellular matrix (ECM) interactions.

Neutrophils Leukocytes responsible by mediating immune responses against infectious microorganisms.

"Outside-in" signaling A process in which ligand binding transduces signals from the extracellular domain to the cytoplasm in the classical outside-in direction.

Pulmonary embolism A condition caused by blood clots that travel to the lungs from the legs or (rarely) other parts of the body.

Vascular disease Circulation disorders that affect blood vessels (arteries and veins).

Venous thromboembolism Condition that includes both deep vein thrombosis and pulmonary embolism.

Introduction

Adhesive Properties of Neutrophils

Neutrophils are considered short-lived cells (<6 days) and comprise of 40–60 % of the leukocyte population in human blood. These cells are the first leucocytes to arrive at sites of infections or injuries. Neutrophils present an elaborate and complex migrating process out of the vascular lumen, where they execute the first efforts of defense, exhibiting functions such as killing and phagocytosis of invading pathogens. Furthermore, neutrophils are involved in the inflammatory response, recruiting other leukocytes by the release of proinflammatory cytokines and chemokines (Voisin and Nourshargh 2013).

Neutrophil recruitment to inflamed vascular endothelium has been described as a multistep process modulated by chemokines, selectins, and integrins that engage in a stepwise manner to initiate intracellular signals and adhesive bond formation (Ley 2002). Studies have been conducted using new methods to evaluate and understand the physiology of the leukocyte migration process, and a general, widely validated model describing the entire process has been generated (Montresor et al. 2012).

Briefly, the process of leukocytes adhesion and transmigration through the endothelial wall consists of chemoattraction and rolling, followed by firm attachment and migration to extravascular tissues (Ley et al. 2007; Woodfin et al. 2010). For leucocytes-endothelial cells attachment, it is necessary that rolling leukocytes become fully resistant to the flow and stop on the vessel wall, which is known as

stable arrest phase and is considered a critical process in leukocytes adhesion mechanisms. A sudden change in integrin avidity mediates this process (Montresor et al. 2012; Takada et al. 2007).

Integrins are cell adhesion receptors expressed on different cell types that participate in cell-cell or cell-matrix interactions. The integrins on the cell membranes exist as heterodimers composed of one α (alpha) and one β (beta) subunit. In humans, at least 24 different heterodimers formed by the combination of 18 α and 8 β subunits have been indentified (Chigaev and Sklar 2012). Each subunit contains a large extracellular domain, a single transmembrane helix, and a short cytoplasmic domain (Hynes 2002).

In the circulation, integrins are normally found in a low-affinity state for the ligands. However, these receptors undergo a structural and topological change to increase its binding efficiency, through spatial rearrangement on the cell plasma membrane. This rearrangement leads to the integrin activation, which is mandatory for the quick arrest of the circulating cells (Montresor et al. 2012).

The step of integrin activation involves bidirectional signals across the cytoplasmic membrane, called inside-out and outside-in signaling pathway (Ginsberg et al. 2005). Wang and Luo (2010) have suggested a model of inside-out activation of integrins that involves the binding of intracellular proteins to the integrin cytoplasmic domains. It has been described that the cellular activator talin mediates the binding of the actin filaments to integrin β subunit cytoplasmic domain, promoting the separation of the integrin α and β helix (Hu and Luo 2013; Tadokoro et al. 2003). After separation, the α subunit helix maintains a similar structure, whereas the β subunit helix is tilted by inserting 5–6 residues into the hydrophobic lipid membrane core. This process of separation of the two transmembrane helices leads to the extension and swing-out of the hybrid domain, resulting in a switchblade-like conformational change of the integrin's extracellular domains; as a result, integrin exhibits a high-affinity state for ligands (Hogg et al. 2011; Schürpf and Springer 2011).

The outside-in signaling pathway requires the binding of integrins to extracellular ligands, which results in a variety of signal transductions across the plasma membrane, which enhance cell adhesiveness (Arnaout et al. 2005; Montresor et al. 2012). Possibly the most important process of outside-in signaling is the lateral mobility. This process initiates with the contact of integrins with extracellular matrix ligands and integrins clustering, which increases ligand binding valency and avidity. This lateral association of integrin heterodimers transfers extracellular information into corresponding intracellular reactions by the recruitment of effectors to the integrin cytoplasmic tail, as well as defines stable connections to the extracellular matrix (Hu and Luo 2013). The outside-in signaling is responsible for the regulation of cell migration, differentiation, proliferation, and survival (Wang et al. 2011; Wang and Luo 2010; Luo et al. 2007; Hu and Luo 2013) (Fig. 1).

Neutrophils express a variety of adhesion molecules on their surfaces that are required for transendothelial migration. The L- and P-selectin mediate tethering and rolling on the endothelium, while firm adhesion is mediated by Very Late Antigen-4 (VLA-4, CD49D/CD29) and by β_2 integrin-complex, as Macrophage antigen-1

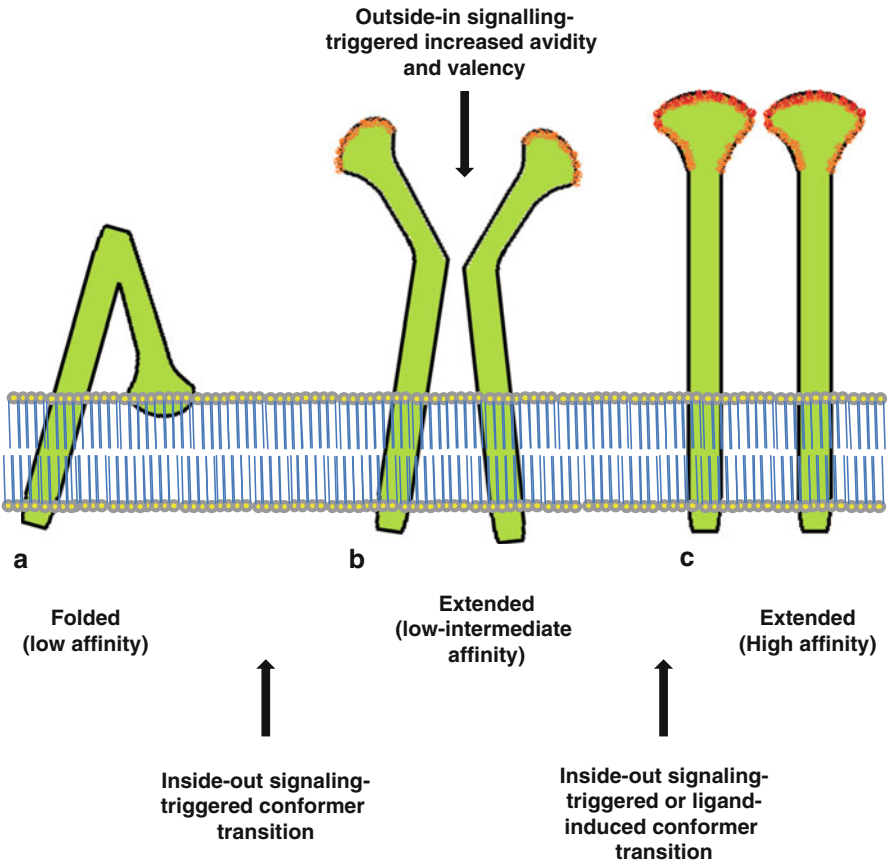

Fig. 1 Integrin activation process Integrin activation involves bidirectional signals across the cytoplasmic membrane, called "inside-out" and "outside-in" signaling pathway. Both mechanisms act together to promote integrin conformational changes and activation. Integrin conformational states differ both in their overall extension over the plasma membrane as well as in the arrangement of their headpiece. (**a**) Resting state (low affinity); (**b**) First step activation involves switchblade-like conformational change of integrin extracellular domains; (**c**) Integrin's high affinity state, with hybrid domain epitope exposed

(Mac-1, CD11b/CD18) and Lymphocyte Function-associated Antigen-1 (LFA-1, CD11a/CD18) (Petri and Bixel 2006).

VLA-4 Integrin exhibits a low-affinity state, bent conformation, with a hidden hybrid domain epitope, in the absence of an extracellular ligand. Recent insights based in small fluorescent ligand-mimicking probes have suggested that the activation process of VLA-4 includes multiple complex molecule conformational states that involve extension of integrin and hidden or exposed hybrid domain epitope (Chigaev and Sklar 2012).

The most detailed description during mechanisms of integrin activation in leukocytes comes from studies of LFA-1 (Montresor et al. 2012). LFA-1 adhesion molecule was one of the first integrins described as a participant of the firm cell adhesion process of activated cells (Chigaev and Sklar 2012).

Recently, it has been demonstrated that LFA-1 may assume at least three distinct conformations, distinct either in their complete extension over the cytoplasmic membrane or in the availability of their headpiece (Montresor et al. 2012; Springer and Dustin 2012; Nishida et al. 2006).

It has been suggested that the extended conformation of LFA-1, with high topographical availability of the ligand-binding headpiece, can also present a low-affinity state (Montresor et al. 2012; Salas et al. 2006). This conformation of low/intermediate-affinity state could enhance the ability of LFA-1 in mediating the rolling process on endotheIial cells; in this situation the affinity of LFA-1 for ICAM-1 (Intercellular Adhesion Molecule I) increases upon the selectin triggering (Montresor et al. 2012; Miner et al. 2008). Importantly, low, intermediate, and high affinity integrins possibly constitute discrete and reversible states in a progression of integrin structural rearrangement (Montresor et al. 2012; Shamri et al. 2005).

Therefore, neutrophils transmigration process can be moderated by the same adhesion molecule existing in different conformers, which can be reversibly controlled through these cellular signaling pathways (Chigaev and Sklar 2012). Furthermore, application of a mechanical force can lead to the stabilization of ligand binding or "catch bond" (Kong et al. 2009), once lateral shear force can notably change the activity state of LFA-1 molecule (Hogg et al. 2011) (Fig. 2).

Accordingly, integrin-initiated outside-in and chemoattractant-triggered inside-out signaling cascades simultaneously to collaborate to the enhanced integrin affinity for the ligand and to the maintenance and prolongation of the arrested leukocytes in circulation (Montresor et al. 2012). Thereby, these phenomena modulate the process of neutrophil adhesion and migration to areas of inflammation, which is important for host defense (Askari et al. 2009).

However, researchers have observed that excessive recruitment of activated neutrophils can cause damage to the host and contribute to the development of inflammatory disorders. Therefore, neutrophils have been implicated in the pathogenesis of both acute (e.g., myocardial infarction) and chronic (e.g., atherosclerosis) vascular inflammatory conditions (Voisin and Nourshargh 2013).

Vascular Disease and Adhesive Properties of Neutrophils

Vascular diseases are multifactorial pathological conditions that represent a major health problem worldwide. Therefore, due to the economic, social, and health impact, early and precise detections of new biomarkers are crucial to identify the exposed population.

Acute coronary events are associated with activated leukocytes and an intense inflammatory response. Moreover, inflammatory pathways promote thrombosis, a

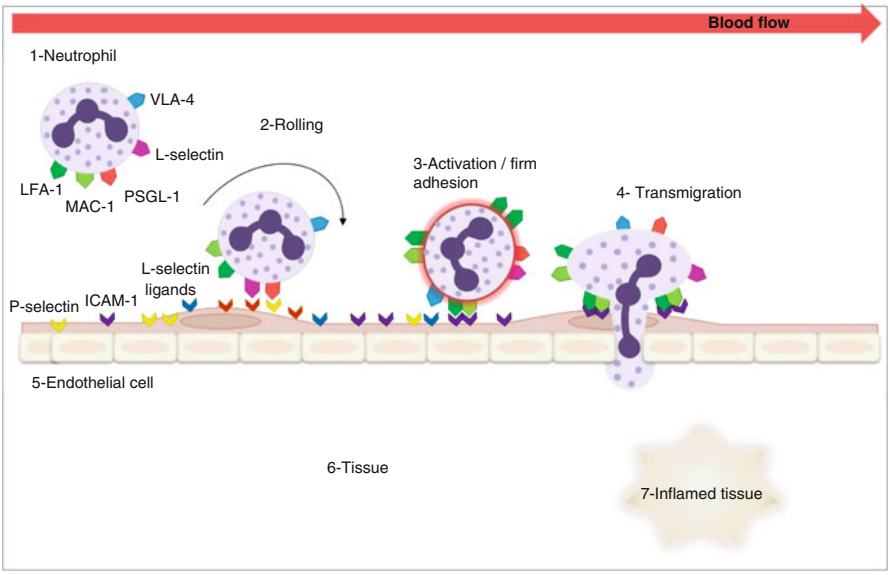

Fig. 2 Neutrophils adhesion and transmigration through the endothelium line Process of (1) leukocytes adhesion and transmigration through the endothelial wall consists of chemoattraction and rolling (*2*), followed by firm adhesion (*3*) and migration to extravascular tissues (*4*). Circulating leucocytes attach to the endothelium by the interaction of leucocytes and endothelium selectins and initiate the rolling process. When the rolling leukocytes become fully resistant to the flow and stop on the vessel wall, a sudden change in integrin avidity may occur and promote a firm leucocyte-endothelial adhesion. The leucocyte-endothelial adhesion is mediated mainly by MAC-1 and LFA-1 interactions with their endothelial ligands (ICAM-1). After the firm adhesion process, neutrophils migrate through the endothelium to the site of inflammation. *ICAM-1*: Intercellular Adhesion Molecule-1, *MAC-1*: Macrophage antigen-1, *LFA-1*: Lymphocyte Function-associated Antigen-1

late and dreaded complication of atherosclerosis responsible for myocardial infarctions and most strokes (Libby 2002).

Studies of necropsies of patients with acute myocardial infarction (AMI) have shown neutrophilic infiltration of necrotic myocardial tissue within the first day of onset of acute myocardial infarction (Swirski 2014). However, the presence of leukocytes in the myocardium requires endothelial transmigration or diapedesis, which is facilitated by increasing the expression of adhesion molecules by endothelial and leukocyte cells (Meisel et al. 1998). Therefore, the increased neutrophil counts during an episode of myocardial infarction could possibly be accompanied by a corresponding increase in the expression of cell-surface adhesion molecules (Meisel et al. 1998).

Meisel and coworkers (1998) evaluated the expression of neutrophil adhesion molecules in patients with AMI, and they observed that the expression of Mac-1 was increased in patients by 133 % ($p < 0.001$) on day 1 compared with age-matched control subjects. In addition, neutrophils isolated from AMI patients showed elevated neutrophil adhesion to endothelial cells compared to those isolated from controls. The treatment with anti-CD11b antibodies significantly reduced

neutrophil adhesion when compared with the untreated control group (Han et al. 2012). The described evidences of changes in the expression of neutrophil cell surface adhesion molecules are important and clinically relevant. Enhanced neutrophil adhesiveness could be involved in the myocardial reperfusion failure after thrombolysis, known as "no-reflow" or "slow-reflow" phenomenon (Gibson et al. 1996), and could also be associated with postinfarction events such as ongoing ischemia and infarction extension. Activated leukocytes exposing adhesion molecules in a high-affinity state could adhere to altered coronary endothelium, culminating in a lesion and alteration in the local vasomotor function, therefore compromising runoff flow (Meisel et al. 1998; Mügge et al. 1991).

The increased neutrophil adhesion in AMI patients can be related to increased inflammation. Peripheral white blood cell (WBC) counts increase significantly after myocardial infarction and are associated with disease severity (Packard and Libby 2008; Chia et al. 2009). In response to inflammatory signals, the adhesion and migration of leukocytes are crucial, and neutrophil adhesion to endothelial cells through adhesion molecules is a central feature of the inflammatory response (Butcher 1991).

Another study showed that ICAM-1-dependent neutrophil adherence plays an important role in reperfusion injury and that neutrophils' adherence and infiltration contribute significantly to coronary endothelial dysfunction (Ma et al. 1992).

Inflammation also plays an important role in acute ischemic stroke. Much of the damage develops gradually over the course of a few hours. It is believed that leukocytes liberate inflammatory cytokines and other neurotoxins in the ischemic brain. Moreover, previous evidence has demonstrated that microvascular occlusion is started through platelet-leukocyte-endothelium interactions in the ischemic penumbra (Alvaro-González et al. 2002; Chamorro 2004; Tsai et al. 2009).

Patients with ischemic stroke demonstrated changes in β_2 integrin expression (Kim et al. 1995). Furthermore, neutrophil adhesion molecules were also evaluated in patients during the stroke onset and on days 7, 30, and 90 post stroke (Tsai et al. 2009). This study highlighted at least three important points. First, the findings showed increased expressions of P-selectin glycoprotein ligand-1 (PSGL-1) of neutrophils in acute stroke patients, and this neutrophil activation persisted for at least 3 months after the onset of cerebral ischemia. The PSGL-1 glycoprotein link to the E-selectin and P-selectin expressed in the surface of endothelium cells; besides, neutrophils can adhere to platelets via PSGL-1/P-selectin interaction. Neutrophil PSGL-1 plays an important role in arterial thrombogenesis by forming stable platelet-leukocyte aggregates (McEver and Cummings 1997). This finding suggests that the persistent activation of circulating neutrophils play a pathophysiological role in the acute and chronic phases following an ischemic stroke. Additionally, this study observed that the expression of Mac-1 on neutrophils is enhanced immediately after the stroke and is normalized during the following months. Thus, it may be hypothesized that circulating neutrophils interact continuously with activated endothelium after acute ischemic stroke. Sustained leukocyte-endothelium interaction after cerebral ischemia may cause substantial inflammatory reaction and lead to secondary injury of potentially salvageable neurons in the penumbra surrounding the

infarct. Finally, neutrophil PSGL-1 expression on day 1 was observed to be significantly higher in patients who develop neurological deterioration (END). Increasing evidence indicates that there is an association between increased risk of reinfarction and inhospital death with high WBC counts, especially neutrophil counts (Fisher and Meiselmann 1994). Consequently, early recruitment-adherent neutrophils after ischemic stroke seem to play an important role in patients with a stroke in progression (Tsai et al. 2009).

The adhesive properties of neutrophils have also been discussed in sickle cell disease (SCD) (Canalli et al. 2011). SCD is characterized by red blood cell sickling, hemolysis, and a chronic inflammatory state in which the leukocyte plays an important role. Microvascular occlusion is responsible for much of the pathophysiology that underlies the clinical manifestations of SCD. Although vascular occlusion is mediated at least in part by the inability of poorly deformable, irreversibly sickled red blood cells to traverse microcirculation, other vaso-occlusive processes have also been implicated (Kasschau et al. 1996). Sickle cell crises are often associated with infection and neutrophil counts are higher in individuals with this disease (Okpala 2004).

Reports suggest that initiation and propagation of a vaso-occlusive event occurs by impaired blood flow due to excessive recruitment of adherent leucocytes to the vascular endothelium and their interactions with circulating erythrocytes (Canalli et al. 2008; Chiang and Frenette 2005).

In addition, studies using intravital microscopy techniques during a flowing inflammatory stimulus demonstrated that leukocytes, particularly neutrophils, of mice expressing sickle haemoglobin adhere to the vascular endothelium and interact with sickle red cells initiating a vaso-occlusive process (Turhan et al. 2002). This data strengthens the hypothesis that neutrophils play a direct role in the sickle cell vaso-occlusion and vascular complications.

Studies in vitro have demonstrated that neutrophils from SCD patients have an increased adherence to endothelial layers compared to control neutrophils, and similar studies showed that SCD neutrophils also display augmented adhesion to integrin ligands such as fibronectin (extracellular matrix component) and ICAM-1 (Fadlon et al. 1998; Kasschau et al. 1996; Canalli et al. 2008). β_2 integrins, particularly the Mac-1, have been reported as highly expressed on the surface of neutrophils from SCD patients in steady state (Lum et al. 2004). Interestingly, Mac-1 expression is further increased in the presence of interleukin-8 (IL-8) (Assis et al. 2005). IL-8 is a chemokine found in high levels in the circulation of SCD individuals, demonstrating that inflammatory environment may further augment altered SCD neutrophil functions (Gonçalves et al. 2001).

Data provided by in vitro investigation indicated that, in healthy individuals, neutrophil adhesions to endothelial cells are mediated mainly by the Mac-1 integrin with a contribution from the LFA-1 integrin, under inflammatory stimulus. On the other hand, under basal and inflammatory conditions, Mac-1, LFA-1 integrin, as well as VLA-4 integrins apparently mediate the adhesion of SCD neutrophils to the endothelium (Canalli et al. 2011). These results suggest that VLA-4 integrins also play a role in SCD neutrophil adhesion to the vascular endothelium.

However, previous data from the same SCD cohort, under similar experimental conditions, showed that neither Mac-1 nor LFA-1 nor VLA-4 surface expressions were significantly altered on nonstimulated SCD neutrophils (Canalli et al. 2008; Assis et al. 2005). LFA-1 and Mac-1 integrins are believed to mediate adhesive interactions via conformational changes, resulting in increased ligand affinity. Thus, these results consistently indicate that increased integrin affinity, rather than significant changes in surface protein expression, bring about the observed increase in adhesive properties of SCD neutrophils.

Despite the VLA-4 integrin low expression in the SCD neutrophil cell membrane, as described previously, this integrin may be found in several conformational states and affinities (Chigaev and Sklar 2012). The exposure of the hybrid domain epitope can also be used to determine VLA-4 ligand binding affinity for unlabeled ligands (Chigaev et al. 2009; Njus et al. 2009). Furthermore, VLA-4 integrin has been implicated in the recruitment of neutrophils during chronic inflammation (Burns et al. 2001; Issekutz et al. 2003), and possibly the inflammatory state associated with SCD stimulates this adhesion molecule on neutrophils.

A recent study using intravital microscopy, in venous thromboembolism (VTE), a disease which comprises of deep venous thrombosis (DVT) and pulmonary embolism (PE), demonstrated that a reduction in blood flow induces a proinflammatory endothelial phenotype that initiates neutrophil recruitment. Recruited neutrophils start fibrin formation via blood cell-derived tissue factor (TF), which is the decisive trigger to the massive fibrin deposition seen in DVT (Saha et al. 2011).

In addition, it was demonstrated in a recent study that an inflammatory profile, expressed by increased adhesion of neutrophils, was associated with a hypercoagulability state in VTE patients, even after the acute DVT episode (between 1 and 6 years after the thrombotic event) (Zapponi et al. 2014). The results could demonstrate a trend toward an increase in the adhesive properties of neutrophils in VTE patients when compared with healthy individuals. Patients were also analyzed in separate groups, and VTE patients with higher D-dimer plasma levels and residual vein occlusion (RVO) presented the highest neutrophils adhesiveness and also had higher levels of circulating inflammatory markers, such as interleukin-6 (IL-6), IL-8, and TNF-α. Interestingly, increased D-dimer levels is a known marker of hypercoagulability (Verhovsek et al. 2008; Tosetto et al. 2012; Carrier et al. 2011; Cosmi et al. 2005; Cosmi et al. 2010). Furthermore, the increase of neutrophils adhesive properties was positively correlated with IL-6 and D-dimer levels, suggesting a possible relationship between these factors.

The scientific community has been discussing the relationship between inflammation and coagulation in the pathogenesis of vascular disease. Evidence points to extensive cross-talk between these two systems, involving platelet activation, fibrin formation and resolution, as well as anticoagulant pathways (Levi et al. 2004).

Inflammation-induced activation of coagulation is a described mechanism believed to be beneficial for host defense in distinct situations. Procoagulant proteins, particularly the tissue factor, expressed by inflammatory cells mediate activation of coagulation cascade and thrombin generation. Thrombin activates platelets and generates platelet-fibrin thrombi. Proinflammatory cytokines may also affect all

these coagulation mechanisms and the natural anticoagulant pathways (Levi and Van der Poll 2010).

Moreover, activated coagulation proteases, anticoagulants, or components of the physiological fibrinolytic system can modulate the inflammatory response through specific cellular receptors on inflammatory and endothelial cells. In addition, the binding of tissue factor-factor VIIa to the protease-activated receptors (PAR-2) results in upregulation of inflammatory responses affecting neutrophil infiltration and proinflammatory cytokine expression (Cunningham et al. 1999).

Therefore, the increased inflammatory markers in VTE patients could enhance the expression of adhesion molecules on endothelial cells (Mihara et al. 2012; Romano et al. 1997; Rincon 2012) and neutrophil adhesion properties, triggering a vicious circle involving inflammation, increased neutrophil adhesion, and activation of coagulation.

In summary, recent studies have supported the hypothesis of an association between inflammation and hypercoagulability, and highlighted the role of neutrophils in this process.

The Applicability of Adhesive Properties of Neutrophils in Clinical Practice

Considerable efforts have been made to achieve the validity and usefulness of diagnostic tests in the interface between clinical medicine and scientific methods. To consider a new method as a potential biomarker, at least four aspects should be evaluated: sensitivity, specificity, predictive value, and prognosis value (Sackett and Haynes 2002).

Sensitivity evaluates whether patients with a target disorder and normal individuals differ regarding test results. As a result, this phase of a diagnostic test evaluation cannot be translated into diagnostic action; however, these studies add biological insights into the mechanisms of disease. As discussed previously, all studies conducted on the adhesive properties of neutrophils in patients with vascular diseases compared with normal individual controls answered the sensitivity issue, highlighting that neutrophil adhesion is higher in patients with vascular diseases.

Specificity of an assay is detected when the test results discriminate between patients and normal individuals. Studies of specificity were not conducted using neutrophil adhesion as a biomarker.

The predictive value of an assay is accessed when specific results predict the disease diagnosis. Studies of predictive value were not conducted using neutrophil adhesion as a biomarker either.

The prognosis value is assessed to evaluate the case where patients who undergo a specific diagnosis fare better (in their ultimate health outcomes) than similar patients who have not been tested (Sackett and Haynes 2002). In this context, neutrophil adhesion may be a marker of poorer prognosis for vascular diseases. At least two studies addressed this question, despite this issue not being their specific goal. A recent study performed by our group, that evaluated the adhesive properties of

neutrophils in VTE patients, demonstrated that patients with increased adhesive profile of neutrophils also presented the highest risk of recurrence of the disease (factors known) (Zapponi et al. 2014). Another study that aimed to assess whether the adhesion molecules of leukocytes could be predictive of the clinical outcomes in patients after a stroke showed that neutrophil PSGL-1 expression was significantly higher in patients with early neurological deterioration (Tsai et al. 2009). Therefore, early recruitment-adherent neutrophils after ischemic stroke seem to play an important role in patients with stroke in progression, as well as in VTE patients.

In conclusion, until now, one cannot assume that the adhesive properties of neutrophils can be used as biomarkers of vascular disease. Nevertheless, the adhesive properties of neutrophils can be viewed as potential biomarkers, as they appear to present some sensitivity and prognostic value to evaluate vascular diseases. Therefore, these results should be validated in other independent studies to arrive at a definite conclusion. In addition, this possible diagnostic test can be associated with a multivariate combination of several other clinical signs detected in medical history, physical examination, or other tests.

Methods to Evaluate Adhesive Properties of Neutrophils

Advances in the understanding of neutrophil adhesive properties have been accomplished using in vitro tools. The first step to evaluate adhesive properties of neutrophils is the isolation of neutrophil cells from the peripheral blood. Neutrophils may be isolated from fresh peripheral blood collected in heparin-containing tubes, using two layers of Ficoll-Paque with different densities (Assis et al. 2005; Zapponi et al. 2014).

Flow cytometry has been widely used in biomedical research. Presently, flow cytometry is used as an additional technique to confirm diagnosis carried out by morphological studies and provides valuable prognostic information. As the number of laboratories with flow cytometry increases, greater quality control, standardization of techniques, and interlaboratory programs will be required (Wu et al. 2010).

Flow cytometry is a powerful analytical tool for the analysis of multiple biological parameters of individual cells or multiple heterogeneous cell populations (Wu et al. 2010). The methodology of flow cytometry enables fast, accurate, and quantitative analysis of cells in suspension. Furthermore, as this is an automatized technique, flow cytometry is one of the methods of choice to evaluate the adhesive properties of neutrophils. As a limitation, the method is costly and demands a technical expert to be performed.

Samples are analyzed by immunofluorescence staining with different fluorochromes, which are excited by the laser. The antibodies bound to the fluorochrome react with the specific antigenic determinants or epitopes on the surface or inside the cells. The initial cell separation step is critical, because it can help eliminate some unwanted populations. Light scattering can be utilized to separate these populations. Cells with more intense fluorescence are those which are tagged with antibodies. Currently, there is a range of antibodies that not only evaluate the expression of

Table 1 Evaluation of neutrophil adhesion molecule expression in vascular diseases by flow cytometry

Study	Disease	Methods	Neutrophil adhesive molecule analyzed	Patients	Controls	*P* value
Tsai et al. (2009)[a]	Ischemic stroke	Flow cytometric (MFI)	PSGL-1 (day 1) PSGL-1 (day 7) PSGL-1 (day 30) PSGL-1 (day 90)	(44.6 ± 1,3) (43.2 ± 1,4) (46.2 ± 1,6) (46.4 ± 1,5)	(39.5 ± 1.816)	*P* < 0.05
			MAC-1 (day 1) MAC-1 (day 7)	(46.3 ± 3.1) (47.9 ± 2.6)	(38.4 ± 2.2)	*P* < 0.05
Meisel et al. (1998)[b]	AMI	Flow cytometric (MFI)	MAC-1 (day 1)	Increased by 133 % in relation to controls		*P* < 0.001
Kim et al. (1995)[c]	Ischemic stroke	Flow cytometric (MFI)	LFA-1 (CD11a)	10.87 ± 10.48	1.44 ± 2.10	*P* < 0.017
			LFA-1 (CD18)	123.78 ± 69.71	88.15 ± 51.60	*P* < 0.05
Kim et al. (1995)[d]	TIA	Flow cytometric (MFI)	LFA-1 (CD11a)	14.55 ± 6.44	1.44 ± 2.10	*P* < 0.017
			LFA-1 (CD18)	172.53 ± 80.21	88.15 ± 51.60	*P* < 0.05

This table summarizes the clinical studies on vascular diseases that evaluated the expression of adhesion molecules of neutrophils, particularly PSGL-1, MAC-1 and LFA-1, by flow-cytometry *AMI* acute myocardial infarction, *MFI* median fluorescence intensities, *TIA* transient ischemic attacks

[a]Values expressed as mean ± SEM

[b,c,d]Values expressed as mean ± SD

adhesion molecules but also their activated epitopes, which facilitates the evaluation of the activated adhesive properties of the neutrophils by flow cytometry. However, in the context of adhesive properties of neutrophils, one of the limitations of flow cytometry is that the integrins' avidity is not taken into account (Tables 1, 2).

Another technique frequently used to evaluate the adhesive properties of neutrophils is the static adhesion assay. The static adhesion assay is a sensitive and versatile in vitro assay, known for mimicking low-shear conditions or interrupted blood flow, which may occur in occlusive vascular events. The main advantages of the static adhesion assay, when compared to other methods of measuring cell adhesion, may be the ability to measure both avidity and affinity of adhesion molecules, examine

Table 2 Evaluation of neutrophil adhesive properties in vascular diseases by static adhesive assay

Study	Disease	Ligand	Relative adhesion (patients/controls)[d]	*P* value
Canalli et al. (2011)[a]	SCD	HUVEC	Increased	$P < 0.05$
Canalli et al. (2008)[b]	SCD	FN/ ICAM-1	Increased	$P < 0.001$/ $P < 0.05$
Zapponi et al. (2014)[c]	VTE	FN	Increased	$P = 0.018$

This table summarizes the results of laboratory studies that evaluated the adhesive properties of neutrophils, from patients with vascular diseases, by static adhesive assays using fibronectin, ICAM-1 or endothelial cells

VTE Venous thromboembolism, *SCD* sickle cell disease, *HUVEC* Human umbilical vein endothelial cells, *FN* fibronectin, *ICAM-1* Intercellular Adhesion Molecule 1

[a]Values expressed as median

[b]Results are expressed as means ± SEM

[c]Values expressed as median

[d]Absolute fold-change values were not available in most of these studies

multiple experimental conditions simultaneously, and it does not require expensive equipment. Another advantage of this method is the ability to detect a small number of cell-adhesion events with accuracy (Bellavite et al. 1992). The intra-assay validation requires that more than 95 % of the cells are viable. In case of significant variability within the same condition, more than three identical replicates should be used. However, this technique is not free of limitations. One potential weakness is the fact that the cell-adhesion events are represented as a percentage, a relative number which may vary from one experiment to another. In order to achieve uniform results, some steps in the protocol must be followed, such as the incubation time and the number of seeded cells which must be uniform among all conditions. Small differences in incubation time may result in inaccurate measurements. It is also important to aliquot exactly the same amount of cells in each well. Another limitation is the fact that this method requires the use of freshly viable cells, and for that the samples should be collected and prepared quickly, which may not be practical in a daily routine. Future improvements of the static adhesion assay may involve automatized cell-aliquot and washing, in order to standardize the process. In addition, an automatized version of the assay would enable the performance of large-scale screening tests (Zapponi et al. 2014).

Conclusions and Perspective

Structural, biochemical, and biophysical studies have greatly contributed to the understanding of the mechanisms of integrin bidirectional signaling across the plasma membrane. Chemoattractant-triggered inside-out and integrin-initiated outside-in signaling events are known to concurrently cooperate to increase integrin affinity for the ligand and to stabilize and prolong the arrest of circulating leukocytes.

This process enables neutrophils to efficiently navigate from the blood stream to inflammatory sites, which is critical for host defense. Nevertheless, the excessive recruitment of activated neutrophils can cause damage to the host and contribute to the development of inflammatory disorders. In this context, studies have emphasized the participation of adherent neutrophils in the pathogenesis of both acute and chronic vascular diseases but more importantly have highlighted an association between neutrophils and disease progression. However, until now, it has not been possible to assume that the adhesive properties of neutrophils could be used as biomarkers of vascular disease, mainly because the methodologies carried out so far are not applicable in a clinical routine, and also due to the lack of validation of the results of studies conducted to date. However, adhesive properties of neutrophils are possibly potential biomarkers, as they seem to present some sensitivity and prognostic value to evaluate vascular diseases.

Summary Points

- This chapter focuses on the adhesive properties of neutrophils as a potential biomarker to evaluate vascular disease.
- The migration of neutrophils from blood to inflamed tissues is modulated by chemokines, selectins, and integrins that initiate intracellular signals and adhesive bond formation.
- The excessive recruitment of activated neutrophils can cause local tissue damage and contribute to the development of inflammatory disorders.
- Neutrophils have been implicated in the pathogenesis of both acute and chronic vascular inflammatory diseases.
- Vascular diseases are disorders of the vascular system that impair the blood flow. These diseases are caused by the formation of clots in the blood stream or by the inflammation of the endothelial tissue.
- Vascular diseases represent major health problems worldwide; therefore, due to the economic, social, and health impact, early and precise detections of new biomarkers are crucial to identify the exposed population.

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Comparing Cardiac Computed Tomography and Histology in Coronary Artery Stenosis

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Sebastian Leschka, Stephan Waelti, and Simon Wildermuth

Contents

Abstract

Coronary computed tomography angiography (CCTA) has become a well-established diagnostic tool for the assessment of coronary artery stenosis. In particular, the high negative predictive value of almost a 100 % in patients with an intermediate likelihood of coronary artery disease permits reliable exclusion of coronary artery stenosis in patients with normal CCTA. Despite of the high diagnostic accuracy, CCTA is able to visualize not only the coronary artery stenosis but the coronary artery plaque. Differentiation of the coronary plaque composition is crucial to identify plaque conditions being prone to rupture. CCTA

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_12

provides a high accuracy for plaque detection and yields important diagnostic information on the characterization of coronary artery plaques and the quantification of coronary artery stenosis.

Keywords

Coronary computed tomography angiography • Coronary artery disease • Plaque imaging • Plaque characterization • Coronary artery stenosis • Histopathology

Abbreviations

CCTA	Coronary computed tomography angiography
CT	Computed tomography
HU	Hounsfield units
IVUS	Intravascular ultrasound

Key Facts of Coronary Computed Tomography Angiography

- Coronary computed tomography angiography has a high diagnostic accuracy for the assessment of coronary artery stenosis particularly to exclude substantial stenosis in patients with atypical chest pain and an intermediate likelihood of coronary artery disease.
- Coronary computed tomography angiography reliable depicts all kinds of coronary artery plaques.
- The depiction rate of coronary computed tomography angiography depends on the observer's experience, the stage of plaque progression, and the presence of calcifications.
- Characterization of coronary artery plaques is feasible by coronary computed tomography angiography and permits identification of vulnerable plaques.
- The quantification of coronary artery stenosis by coronary computed tomography angiography correlates with that from quantitative catheter angiography, but grading systems for the degree of coronary artery stenosis are recommendable for practical reasons.

Definitions

Cardiac computed tomography Cardiac computed tomography is a diagnostic tool in which data acquisition of a computed tomography system is synchronized to the heartbeat by the ECG signal in order to suppress motion artifacts by the beating heart.

Catheter coronary angiography Catheter coronary angiography is a cardiology diagnostic test and procedure in which a catheter is placed in the coronary artery and the heart chambers to visualize the coronary artery lumen and the blood flow.

Dual-energy computed tomography Dual-energy imaging is a special computed tomography imaging method which is supplied by systems from different vendors. The cornerstone of dual-energy imaging is to perform computed tomography at two different energy levels. As a result, materials can be differentiated by differences in Compton scattering and photoeffect at both energy levels, and therefore dual-energy imaging adds to the diagnostic information from computed tomography imaging.

Dual-source computed tomography Dual-source computed tomography is a special system, in which in opposition to the traditional single-source computed tomography, the scanner is composed of two X-ray tubes and two detectors are arranged in a 90° angular offset. The benefit of this special arrangement is a substantial increase in temporal resolution and the ability to perform dual-energy imaging.

Intravascular ultrasound Intravascular ultrasound is an invasive cardiological method in which a special catheter with an ultrasound probe attached to the end of the catheter is placed in the coronary artery in order to visualize diseases in the coronary artery wall and visualization of coronary artery plaques.

Stary classification scheme The Stary classification scheme is a histopathological grading system for atheromatous plaques, which has been proposed by an American Heart Association consensus group headed by Herbert Stary. This scheme divides the coronary artery plaques by the different plaque components into eight different types and differentiates early from advanced-stage plaques.

Atherosclerotic cardiovascular disease is one of the leading causes of morbidity and mortality in developed countries. Coronary artery disease is characterized by the development of plaques due to the accumulation of lipid or calcified deposits in the coronary artery wall (Enrico et al. 2009; Olgac et al. 2009). Acute myocardial infarction is most frequently the result of rupture of coronary atherosclerotic plaque with subsequent formation of an intraluminal thrombus (Arbustini et al. 1999; Thim et al. 2010; Henzler et al. 2011; Precht 2013). Procedures that can improve the detection, the determination of the plaque composition, and the plaque quantification are essential. Traditionally, imaging of coronary artery disease was based on catheter coronary angiography. Unfortunately, this method is limited by the luminographic visualization of the coronary arteries and the inability to visualize coronary atherosclerotic plaques (Fig. 1) (Raggi et al. 2005). Therefore, intravascular ultrasound (IVUS) is considered as the clinical reference modality for most accurate characterization and detection of coronary atherosclerotic plaques, regarding plaque morphology and quantification of arterial stenosis (Raggi et al. 2005). Despite its advantages compared with catheter coronary angiography, the application of IVUS for risk stratification in larger patient populations is limited due to its invasive nature. Hence a noninvasive, widely available technique for the detection and characterization of the content of coronary atherosclerotic plaques is desired. Today, computed tomography (CT) is the preferred cross-sectional imaging modality for detection, characterization and quantification of coronary artery stenosis.

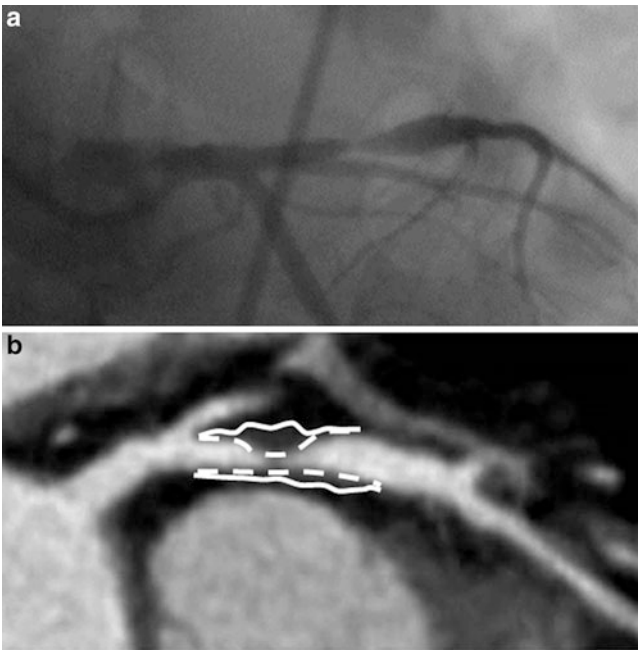

Fig. 1 Catheter angiography and CCTA in a 46-year-old male with atypical chest pain. Catheter angiography (**a**) demonstrates the substantial coronary artery stenosis in the left ascending artery. CCTA (**b**) is able to visualize the coronary artery stenosis and its cause, the atherosclerotic plaque

Coronary Plaque Detection with CT

Technical Requirements

Coronary computed tomographic angiography (CCTA) is nowadays a clinically accepted technique for noninvasive and accurate assessment of coronary artery stenosis and coronary plaques (Dettmer et al. 2013; Alkadhi et al. 2008; Leber et al. 2006; Leschka et al. 2005). CCTA not only permits noninvasive detection and quantification of coronary artery stenosis but also enables the visualization and characterization of the atherosclerotic plaque (Fig. 1) (Feuchtner et al. 2012; Dey et al. 2010)

Initial CT studies using conventional single-energy CT systems with 4-slice and 16-slice technology have been limited by the relatively low spatial and temporal resolution (Becker et al. 2003; Halliburton et al. 2006). In 2004, 64-slice CT systems have become available for the first time and proofed to be robust enough for cardiac CT imaging in daily clinical practice (Fig. 2). These CT systems allow for an examination of the entire heart within a short breath-hold time of less than 10 s. Due to improvements in spatial resolution, 64-slice CT systems yield a detailed

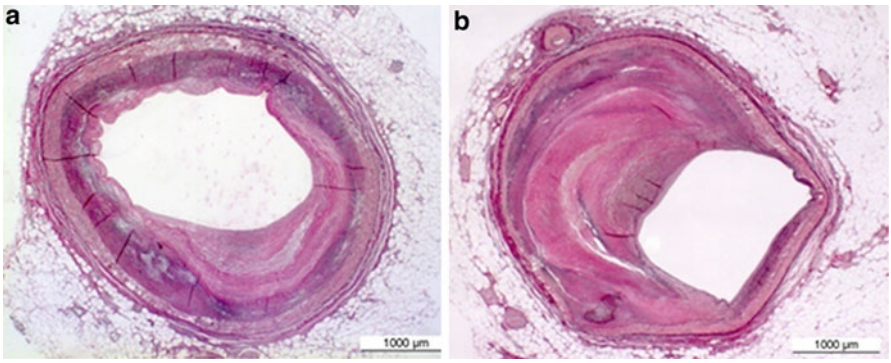

Fig. 2 Histological sections of a Stary type V (**a**) and a Stary type VIII (**b**) coronary atherosclerotic plaque

evaluation of small-sized branches of less than 1 mm in diameter (Leber et al. 2005a; Leschka et al. 2005; Mollet et al. 2005; Raff et al. 2005; Ehara et al. 2006; Nikolaou et al. 2006; Ong et al. 2006; Pugliese et al. 2006; Ropers et al. 2006). However, despite the improved temporal resolution, 64-slice CT systems still need a low and stable heart rate for optimal image quality (Leschka et al. 2006), rendering a heart rate control by the means of beta-blockers prior to CCTA frequently required. The remaining challenges of temporal resolution and a resulting high detector coverage speed have led to the development of the latest CT systems: 256-/320-slice CT and dual-source CT. These CT systems are capable of covering the entire heart within a single heartbeat. While the 256-/320-slice CT systems mainly focus on a large detector coverage, the dual-source CT system mainly focuses on a high temporal resolution: it is composed of two X-ray tubes, and two corresponding detectors are mounted onto the rotating gantry with an angular offset of 90° (Flohr et al. 2006) thus reducing the rotation angle needed to acquire the required projection data for image reconstruction from half a rotation in a single-source CT to a quarter of a rotation in a dual-source CT and thereby almost doubling the temporal resolution (Flohr et al. 2006; Leschka et al. 2007; Matt et al. 2007).

A more detailed chronology on the technical development of CT for cardiac imaging could be found in a recent review article (Leschka et al. 2009).

Depiction Rate of Coronary Artery Plaques with CT

A huge number of studies investigated the diagnostic performance of CCTA in the detection of coronary artery stenosis in comparison to catheter angiography (Table 1) (Leschka et al. 2009). All of the studies, irrespective of the CT scanner type used, demonstrated a high negative predictive value of CT approaching 100 %, but the rate of not-evaluative coronary artery segments highly depends on the CT system used with up to 32 % of coronary artery segments being not-evaluative when using 4-slice CT (Morgan-Hughes et al. 2003) and as low as 0 % being not-evaluative on 64-slice

Table 1 Diagnostic performance of CCTA the detection of coronary artery plaques in different 16-slice and 64-slice CT studies

CT system studies	Patients (n)	Calcified plaque		Non-calcified plaque	
		Sens. (%)	Spec. (%)	Sens. (%)	Spec. (%)
16-slice CT					
Achenbach et al. (2004)	22	94	94	53	87
Leber et al. (2005)	37	95	92	78	92
Caussin et al. (2005)	22	100	93	81	80
64-slice CT					
Leber et al. (2005)	19	95	94	83	94
Sun et al. (2008)	26	93	90	97	–
Petranovic (2009)	11	86	89	90	–

CT (Leschka et al. 2005) and dual-source CT (Tsiflikas et al. 2009). In general, the average specificity for patients with significant coronary artery stenosis has increased, while the number of not-evaluative segments decreased with modern CT technology. In addition, coronary artery plaques in proximal and mid-segments are depicted with a higher sensitivity than in distal segments (Leschka et al. 2009).

Despite of these encouraging results on the high diagnostic performance of CCTA, one must consider that those clinical studies commonly center on the detection of substantial coronary artery stenosis (i.e., luminal diameter narrowing of more than 50 %), while the CT detection rate of early stage coronary plaques causing a non-substantial coronary artery stenosis which may represent precursors of future coronary artery stenosis is less frequently investigated.

In a phantom study using a 64-slice dual-source CT, the detection rate for coronary plaques of any histopathologic subtype was 79 %, while the detection of advanced-stage plaques was 100 %. Nevertheless, although the CT system used in the mentioned study (Leschka et al. 2010) had a spatial resolution of 0.4 × 0.4 × 0.4 mm^3, the size of early stage atherosclerotic plaques still was below the resolution of the CT scanner, so none of the very early stage plaques (i.e., Stary I) and only 17 % of Stary II plaques could be identified by CT.

Influence of Observer Experience on Plaque Detection with CT

An important factor affecting the reliability and accuracy of coronary artery plaque detection with CCTA is the observer's experience in cardiac imaging. Saur et al. (2010) conducted a study involving three readers with different levels of expertise in cardiac CT imaging. All three readers evaluated 50 CCTA data sets twice regarding the presence or absence of coronary artery plaques. Afterward, a consensus reading was performed in which all three readers jointly determined the presence of coronary artery plaques, which was then defined as the reference standard of the study. The observer's variability for plaque depiction varied between $\kappa = 0.582$ for the least experienced reader and $\kappa = 0.892$ for the observer with the

highest experience in cardiac imaging. Furthermore, with an increasing level of experience, intraobserver variability for coronary plaque detection and the time required for interpreting the cardiac CT study significantly decreased.

> The accuracy of coronary artery plaque detection depends on the observer's experience, and the intraobserver variability and evaluation time decrease with increasing observer experience (Saur et al. 2010).

Coronary Plaque Characterization with CT

Data from patients with acute coronary syndrome have shown that two-thirds of culprit plaques were angiographically non-obstructive (i.e., less than 50 % luminal stenosis) (Ambrose et al. 1985). As such, the composition of the atherosclerotic lesion rather than the degree of stenosis is currently considered to be the most important determinant for the biomechanical stability of coronary atherosclerotic lesions and for acute clinical events (Pasterkamp et al. 2000). Albeit coronary calcification is associated with worse cardiovascular prognosis, the presence of calcification does not decrease the biomechanical stability of the plaque (Huang et al. 2001). In contrast, a thin fibrous cap and a large lipid pool are important determinants of increased risk for plaque rupture (Huang et al. 2001). The ability to recognize such plaques vulnerable to rupture is important for the detection of this risk and the possible prevention of acute coronary events.

In CCTA, plaque elements can be characterized by their CT attenuation profiles, which are determined by the physical properties of the contained elements (atomic number and density) and by the delivered tube voltage and tube current. A CT classification of coronary plaque structures has been performed according to the presence or absence of calcifications (Achenbach et al. 2004), by density measurements of the plaque (Nikolaou et al. 2003), or by both methods combined (Kopp et al. 2001; Becker et al. 2003; Schroeder et al. 2004a; Halliburton et al. 2006).

Histopathological Classification

Atherosclerotic lesions are often classified according to Stary (grades I–VIII) in histopathology (Stary 2000). An atherosclerotic plaque is classified as type I when containing small isolated groups of macrophages with intracellular fat accumulation; as type II when multiple foam cell layers are formed; as type III when isolated extracellular lipid pools are present; as type IV when the predominant tissue in the plaque is fat (histologically known as lipid core) and there is no detectable fibrotic component located toward the lumen; as type V when the predominant tissue in the

plaque is fat with a fibrotic plaque component located toward the lumen (histologically known as fibrous cap) (Fig. 4); as type VI when the lumen adjacent to the plaque contains thrombotic material or the plaque surface is interrupted, indicating a complicated plaque; as type VII when the predominant tissue of the plaque is calcified; and as type VIII when the non-calcified tissue component is predominantly fibrotic (Leschka et al. 2010).

The plaque composition determines the risk of acute coronary events (Motoyama et al. 2007).

CT Classification of Coronary Plaques According to Calcified Components

The qualitative approaches classify coronary plaques as non-calcified, mixed, or calcified (Becker et al. 2003; Achenbach et al. 2004) (Fig. 3) or as predominantly lipid rich, intermediate, or predominantly calcified (Schroeder et al. 2004a). Becker

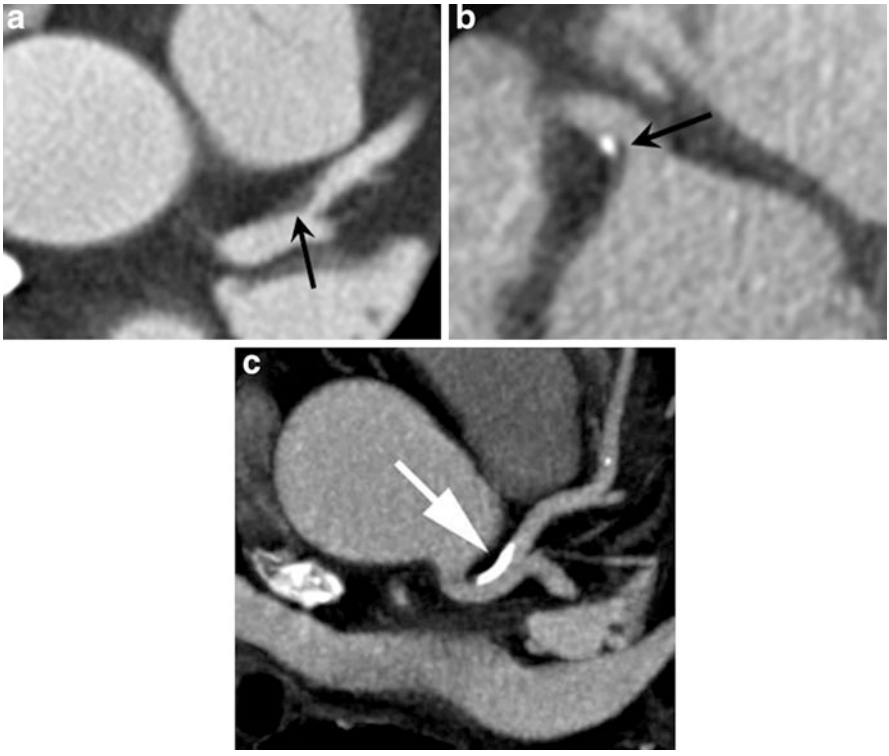

Fig. 3 Qualitative classification of coronary artery plaques in CCTA as non-calcified (**a**), mixed (**b**), and calcified plaques (**c**)

et al. (2003) reported a moderate correlation of this descriptive classification scheme compared to the histopathological Stary classification. In particular, calcified plaques were found even in early stage Stary type III plaques. Moreover, plaques were classified as being non-calcified by CT, although being predominantly calcified type VII plaques. In contrast, Leschka et al. (2010) reported that none of the Stary type VII plaques were classified as non-calcified. Mixed plaque composition was found in Stary type IV–VIII and in 15 % of type VII plaques, whereas purely calcified plaques were not only present in type VII but also in type V and type VI plaques. Non-calcified plaques were found in all Stary types with the exception of type VII plaques. Thus, a qualitative CT classification as non-calcified might be useful to identify early stage plaques, whereas CT classification as mixed or calcified is highly predictive of advanced plaque stage. Nevertheless, a descriptive classification that is based on the presence of calcified components oversimplifies the histologic structure of coronary artery plaques and does not permit the identification of vulnerable plaques such as the fibrous cap atheroma (Huang et al. 2001; Leschka et al. 2010).

Classification of Coronary Plaques According to Density

The quantitative CT classification scheme for characterization of plaques is based on density measurements for distinguishing lipid, fibrotic, and calcified components (Kopp et al. 2001; Becker et al. 2003; Nikolaou et al. 2003; Schroeder et al. 2004b; Halliburton et al. 2006). A CT density threshold of 60 HU for distinguishing between predominantly lipid-rich and intermediate plaques and of 120 HU for distinguishing between fibrotic and calcified components has been proposed (Schroeder et al. 2004b).

> Areas of plaque with density values <60 HU as measured by coronary computed tomography angiography are associated with an increased likelihood of lipid-core plaque in histology (Puchner et al. 2014).

Unfortunately, the measurement of plaque attenuation can be affected by several factors. Plaque density measurements can also be compromised by the presence of iodinated contrast agent in the arterial lumen (Cademartiri et al. 2005; Halliburton et al. 2006; Pohle et al. 2007). Image noise can compromise the mean HU values particularly of non-calcified plaque components, preventing accurate discrimination of lipid and fibrotic tissues on the basis of density measurements (Barreto et al. 2008). As a consequence, there is a large overlap of CT density values of lipid-rich and fibrous coronary artery plaques in CT, and discrimination of both plaque types by CT density is – if at all – only valid when considering a lipid-rich plaque at a CT density of less than 30 HU (Fig. 4).

In a recent study using dual-source CT (Leschka et al. 2010), only the predominantly calcified plaques of Stary type VII could be clearly distinguished from other plaque types due to the high atomic number of calcium. The average CT density of

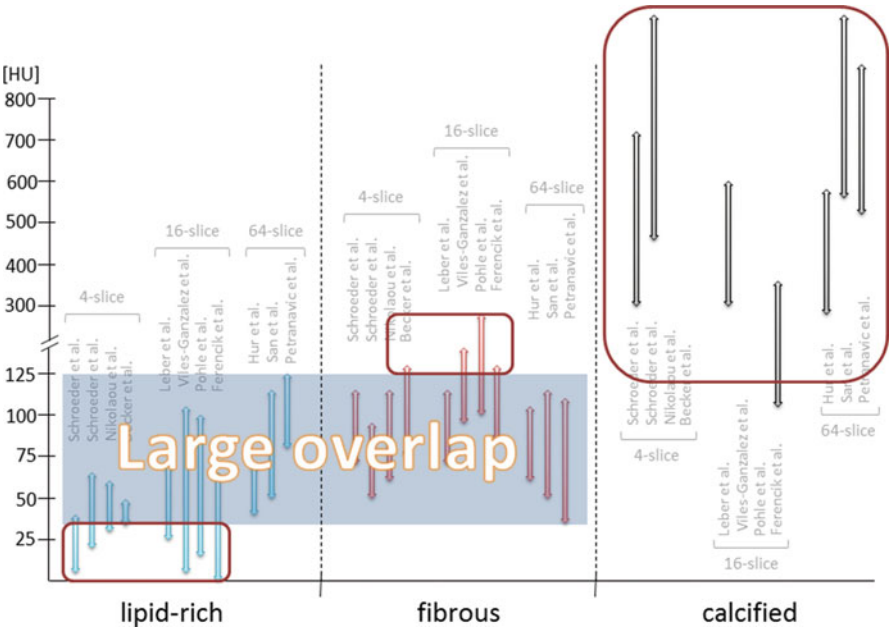

Fig. 4 Different studies on CT density measurements of lipid-rich, fibrous, and calcified coronary artery plaques. There is a substantial overlap of attenuation ranges of lipid-rich and fibrous plaques

early stage Stary type II–III plaques was significantly lower than that of advanced-stage Stary IV–VIII plaques due to the increase of calcified components in advanced-stage plaques with a corresponding increase in the average CT density. However, a differentiation of Stary plaques except the calcified Stary VII plaques by the CT density value is not feasible.

A qualitative CT classification of plaque composition differentiating non-calcified, mixed, and calcified components as well as a quantitative classification that is based on the CT density measurements distinguishes between early and advanced-stage plaques (Leschka et al. 2010).

CT classification of coronary artery plaques on the basis of the presence of calcification and average CT density allows for the identification of Stary type VII plaques with a high diagnostic accuracy (Leschka et al. 2010).

Identification of Vulnerable Plaques by CT

The accurate assessment of plaque composition is of utmost importance, because atherosclerotic plaques that are prone to rupture typically have a lipid-rich necrotic core and a thin fibrous cap (Mann and Davies 1996; Virmani et al. 2000, 2002, 2003,

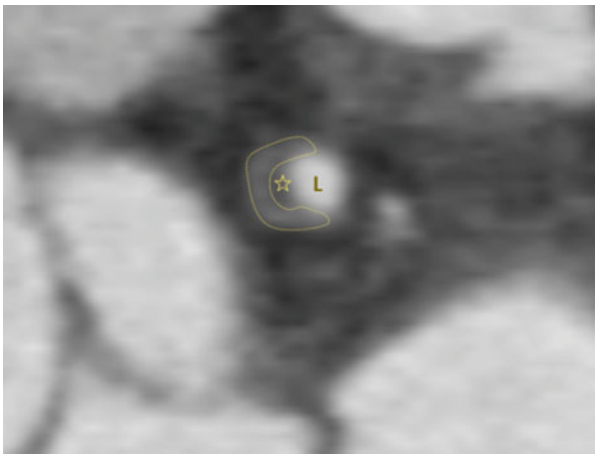

Fig. 5 Cross-sectional CT image of a coronary atherosclerotic plaque with napkin-ring sign. The plaque contains a lipid-rich necrotic core (*asterisk*), while the outer portion of the plaque (*line*) contains a significant amount of fibrous tissue. *L* vessel lumen

2006). Studies using single-source CT scanners found a significant overlap of the attenuation ranges of necrotic cores of rupture-prone plaques and of fibrous plaques, which limited the results (Sun et al. 2008; Hur et al. 2009).

Studies have described a napkin-ringlike attenuation pattern of atherosclerotic plaques on coronary CT images in patients with acute coronary syndrome, potentially representing a culprit lesion. The pattern is characterized by a core with low CT attenuation surrounded by a rim-like area of higher CT attenuation (Tanaka et al. 2008; Goldstein et al. 2009; Kashiwagi et al. 2009; Narula and Achenbach 2009; Maurovich-Horvat et al. 2010). Histopathologically, the lesion is characterized by a necrotic core, which is consistent with the low attenuation core of the plaque, and a significant amount of fibrous plaque tissue, which is consistent with the high attenuation rim on CT images (Fig. 5).

The napkin-ring sign is considered a CT signature of high-risk coronary atherosclerotic plaque (Maurovich-Horvat et al. 2010).

CT images have been reconstructed using filtered back projection algorithms since the inception of the modality. In recent years, iterative reconstruction algorithms have become popular and have been implemented in CCTA. Iterative reconstruction is based on multiple iteration steps taking into account the scanner geometry and noise statistics, and it provides improved image reconstruction with reduced scatter noise at the cost of higher computation time or it may be used to reduce the radiation exposure of CCTA. Several studies have shown that iterative reconstruction algorithms can improve image quality and reduce the radiation dose without increasing the image noise level (Moscariello et al. 2011; Scheffel et al. 2012; Schuhbaeck et al. 2013). It could be demonstrated that iterative reconstruction algorithms may improve the feasibility and accuracy of plaque and stenosis quantification (Moscariello et al. 2011; Scheffel et al. 2012; Fuchs et al. 2013;

Morsbach et al. 2013; Schuhbaeck et al. 2013). In 2014 Puchner et al. (2014) investigated the effect of different iterative reconstruction algorithms on the assessment of atherosclerotic plaque composition, using the histopathologic plaque classification as reference standard. They could demonstrate an improved diagnostic accuracy of iterative reconstruction algorithms for the detection of vulnerable atherosclerotic plaques with a lipid-rich core (Puchner et al. 2014).

Iterative reconstruction improves detection of high-risk lipid-core plaques and thus may lead to improved management and risk stratification of patients with coronary artery disease (Puchner et al. 2014).

One future approach for coronary plaque characterization might be the use of dual-energy CT (Obaid et al. 2014). In dual-energy CT, both tubes of the dual-source CT system are operated with different tube voltage thereby allowing a material differentiation by the means of differences in photo effect and Compton scattering (Johnson 2012). Obaid et al. (2014) demonstrated that the lipid-rich necrotic core has a lower atomic number than calcium in the plaque and a lower density than fibrous tissue and results in both the lowest attenuation values and the smallest change in attenuation at different energies (Obaid et al. 2014). Using the attenuation at two different energies, it turned out that the dual-energy index of a necrotic core is significantly lower than that of fibrous plaques and calcified plaques, and, importantly, there is no significant overlap in the dual-energy indices of the necrotic core and the fibrous plaque (Obaid et al. 2014). There is a significant change in attenuation of the fibrous plaque at different X-ray energies but not of the lipid-rich necrotic core (Fig. 6) (Obaid et al. 2014).

Dual-energy coronary CT angiography imaging may improve differentiation of necrotic core and fibrous plaque (Obaid et al. 2014).

Coronary Plaque Quantification with CT

Quantification of coronary artery stenosis by CT can be performed as diameter stenosis or as area stenosis (Fig. 7). In our experience, diameter stenosis in CCTA correlates better with the invasive quantitative coronary angiography, whereas area stenosis in CCTA correlates better with intravascular ultrasound and histopathology. Some studies have tried to use a quantitative approach for determining percent stenosis and comparing these absolute values to quantitative coronary angiography (Cury et al. 2005, 2006; Hoffmann et al. 2005; Dragu et al. 2006; Busch et al. 2007; Achenbach 2008). Although the degree of stenosis detected by CCTA and conventional invasive angiography correlated, the relationship showed substantial scatter and limits of agreement typically ranged from 20 % to 40 % (Cury et al. 2005, 2006;

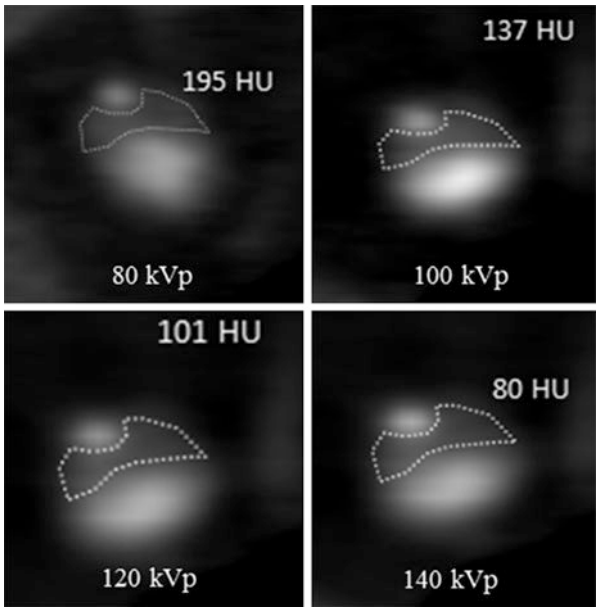

Fig. 6 The CT density of a Stary type VIII plaque (fibrotic, *dotted region*) at different tube voltages (80–140 kVp) substantially decreases at higher X-ray energies

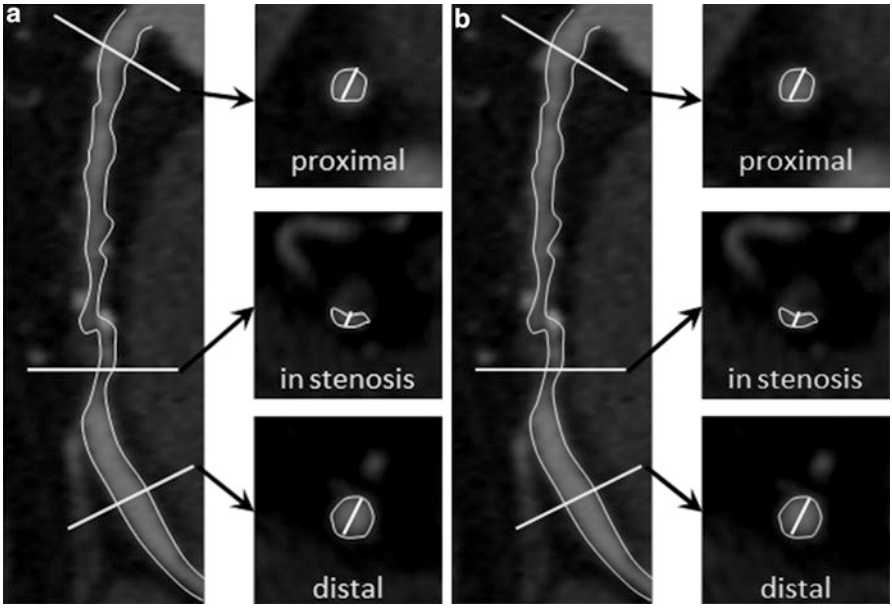

Fig. 7 Schematic drawing of stenosis measurement by CT. (**a**) Diameter stenosis is quantified by measuring the minimal luminal diameter in the stenosis and comparison with the average diameter of normal-appearing coronary segments proximal and distal to the stenosis. (**b**) Area stenosis is quantified by measuring the luminal area in the stenosis and comparison with the average luminal area of normal-appearing coronary segments proximal and distal to the stenosis

Table 2 Different grading systems for luminal diameter stenosis in CCTA

Cheng et al. (2008)		Cury et al. (2006)		Goldstein et al. (2007)	
Grade	Luminal diameter stenosis (%)	Grade	Luminal diameter stenosis (%)	Grade	Luminal diameter stenosis (%)
1	<25	Mild	0–40	1	1–25
2	25–49	Moderate	41–70	2	26–50
3	50–69	Severe	71–100	3	51–70
4	70–89			4	71–99
5	≥90			5	100

Hoffmann et al. 2005; Dragu et al. 2006; Busch et al. 2007; Achenbach 2008). Therefore, applying a grading system for coronary artery stenosis is recommendable for practical reasons (Table 2). Cheng et al. (2008) proposed the use of a categorical 5-point score to grade the stenosis in CCTA and could demonstrate a significant agreement with quantitative conventional invasive coronary angiography. Cury et al. (2006) used a 3-point score system with a wide category of moderate stenosis, ranging from 41 % to 70 %. In daily clinical practice, we prefer the classification proposed by Goldstein et al. (2007) who included a separate category for total coronary occlusion, which can be an important distinction from high-grade stenosis.

Quantitative catheter angiography is the gold standard for coronary artery stenosis quantification. Early reports on stenosis grading in CCTA using 64-slice CT in comparison to catheter angiography demonstrated only a minor correlation between both modalities (Leber et al. 2006). Modern CT systems such as dual-source CT provide accurate correlation of stenosis degree using angiography as reference and are more reliable in the prediction of high-grade lesions reaching >70 % stenosis (Dragu et al. 2008).

Dettmer et al. (2013) investigated the influence of the histopathological plaque type on the stenosis degree grading in CCTA. They found that the overall stenosis degree measurement significantly correlated between CT and histology and that the luminal narrowing of non-calcified plaques is underestimated while the stenosis caused by calcified plaques is overestimated by CCTA.

CT systematically overestimates the degree of stenosis in calcified plaques and underestimates the degree of stenosis in non-calcified plaques, while quantification is accurate in mixed plaques (Dettmer et al. 2013).

Schroeder et al. (2004a) correlated the histopathological Stary classification of atherosclerotic plaques with 16-slice CT, but a classification beyond the three-tier system non-calcified, mixed, and calcified was not possible and the degree of stenosis was not assessed. Dettmer et al. (2013) using a 64-slice dual-source CT found no differences in stenosis degree measurements between

CT and histopathology for the Stary type III–VI and type VIII plaques, while they found a significant difference between the two modalities for the early non-calcified lesion Stary type II and the heavy calcified lesion Stary type VII. The stenosis degree measured by CT was significantly overestimated for Stary type VII plaques (mean difference $-9\ \% \pm 10\ \%$) and significantly underestimated for Stary type II plaques (mean difference $-14\ \% \pm 9\ \%$). The underestimation of early lesions is due to the limited spatial resolution, while it is known that calcifications lead to an overestimation of a stenosis. Both histopathology and CT found a significantly higher stenosis degree in advanced-stage plaques (Stary type IV–VIII) compared to early stage plaques (Stary II and III).

Modern CT reliably depicts advanced-stage coronary artery plaques with an overall good correlation of stenosis degree compared to histopathology. However, the degree of stenosis is systematically overestimated in calcified plaques and underestimated in non-calcified plaques.

Quantification of stenosis degree by CT is only accurate for mixed plaques (Dettmer et al. 2013).

Conclusion

Owing to technical developments in the past years, CCTA has advanced from a research tool to that of an increasingly used diagnostic modality in clinical practice. Modern CT systems not only permit accurate detection and quantification of coronary artery plaques but visualize the underlying coronary artery plaque and provide information on the plaque composition. From the clinical point of view, CCTA may be particularly useful for depiction of vulnerable plaques. Novel developments such as dual-energy CT may improve the detection of rupture-prone plaques.

Potential Applications to Prognosis, Other Diseases, or Conditions

The noninvasive identification of coronary atherosclerotic plaques and the determination of the plaque composition are some of the ultimate goals of coronary imaging. Improvements in cardiac CT imaging have rendered this imaging modality to a fundamental diagnostic tool in cardiology and can significantly improve the risk stratification of patients with suspected coronary artery disease and may improve the prognosis of coronary artery disease by early and focused therapy (Mowatt et al. 2008; Gao et al. 2011).

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Ultrasonic Measurement of Blood Flow Velocity and Applications for Cardiovascular Assessments

44

Gregory R. Bashford

Contents

Abstract

The cardiovascular system is singular among the physiological systems in the combination of its continuous, unceasing functionality, very rapid response to external and internal stimuli, and potential for bodily harm if either of the first two is dysfunctional. Therefore, the ability to quickly and accurately monitor blood flow is an important tool for clinicians. Ultrasound has proved to be uniquely suitable for blood flow monitoring for several reasons, including its relatively low expense, safety due to the use of nonionizing radiation, and ease of portability. However, the most important feature is its real-time feedback, which matches the rapidity with which cardiovascular conditions may change. Ultrasound may be

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_18

used to measure blood flow velocities within most physiological systems noninvasively with immediate visual and aural feedback.

The distinctive advantages of ultrasound permit the assessment of a broad range of cardiovascular function, both healthy and pathological. The main method by which ultrasound measures flow is through a velocity estimate, which is conducted by comparing the phase differences of successive pulse-echo signals from moving tissue or blood. Other novel methods of flow detection using ultrasound have been conceived over several decades, leading to greater usage, accessibility, and clinical adoption. The most recent use of the basic mechanism of blood (or tissue) motion detection is in providing functional information, namely, biomechanical properties, of human tissue for noninvasive assessment.

Keywords

Ultrasound • Blood flow detection • Cardiovascular pathology • Color flow imaging • Spectral Doppler

Abbreviations

AV	Arteriovenous
CDI	Color Doppler imaging
CW	Continuous wave
DTI	Doppler tissue imaging
kHz	Kilohertz
MHz	Megahertz
PRF	Pulse repetition frequency
PW	Pulsed wave

Key Facts of Ultrasound

- It takes about a tenth of a millisecond for ultrasound to travel from the transducer to the heart and return (about twice as long to reach the back wall of the heart and return)
- An acoustic wave traveling through the body can have pressure peaks up to about 50 times as great as atmospheric pressure, at a single point, lasting less than a microsecond. This is not harmful to the body.
- The actual particle (molecular) oscillatory displacement supporting the acoustic wave peaks from about 0.1 to 3 nm – much less than the molecule size.
- Sound speed in different tissues differs over about a 10 % range (although its standard deviation is only a few percent). However, the ultrasound machine does not "know" this and must assume a value – by convention, commercial machines use 1,540 m/s. This means all images are slightly aberrated.
- Until recently, the upper frequency limit of any animal hearing was thought to be shared by dolphins and bats at about 150–200 kHz. In 2013, a study in the journal *Biology Letters* showed the "greater wax moth" capable of hearing close to

300 kHz. To date, no animal is known to be able to hear diagnostic ultrasound frequencies (about 2–12 MHz).

Definitions

A-line The resulting data from one pulse-echo or one transmit-receive process.

Aural Related to the sense of hearing. In Doppler ultrasound, the aural representation of blood flow detection is presented to the clinician by playing the Doppler frequencies through a speaker.

Distal In anatomy, refers to a relative location, meaning "away from" a central or reference point (usually the center of the body). In this chapter, also refers to downstream (e.g., from a stenosis) in the normal direction of circulation.

Lumen The interior of a blood vessel.

Modality A particular type of imaging system. X-ray, computed tomography (CT), magnetic resonance imaging (MRI), and ultrasound are all different imaging modalities.

Piezoelectric A material which transduces mechanical strain to an electric field due to alignment of electric dipoles in the material and vice versa.

Proximal In anatomy, refers to a relative location, meaning "closer to" a central or reference point (usually the center of the body). In this chapter, also refers to upstream (e.g., from a stenosis) in the normal direction of circulation.

Pulse-echo A form of ultrasound transmission and reception where one transducer temporarily emits a short burst of sound and then switches to receive mode (using the dual piezoelectric property) to transduce reflected echoes. Contrast transmit-receive.

Stenosis Any abnormal narrowing or constriction of a passage through the body. In this chapter, stenosis normally refers to a partially obstructed blood vessel due to disease.

Transmit-receive The normal transducer operation of diagnostic ultrasound. A transmit transducer is used to emit sound and a receive transducer is used to transduce detected sound, either from reflection or from transmission through a medium. The transmit and receive transducer may be the same if the emitted sound is a short burst – if so, then transmit-receive is equivalent to *pulse-echo*.

Vasoconstriction A decrease in the diameter of a blood vessel, normally caused by a regulatory system in the body.

Vasodilation An increase in the diameter of a blood vessel, normally caused by a regulatory system in the body.

Introduction

Delivery of oxygen to body tissue is one of the most time-sensitive functions of the major physiological systems. If the body is subjected to a sudden, unexpected loss of oxygen (as would occur in cardiac arrest), brain damage occurs and bodily functions begin to cease after only a few minutes (Choi and Rothman 1990). Sudden obstruction in a blood vessel can cause irreversible tissue death; the amount of tissue loss depends on the location and size of the blood vessel. In addition, all human tissues and organs are affected by long-term deficiency of oxygen (hypoxemia), which can occur in a wide variety of disease states (e.g., pneumonia, congenital heart disease, emphysema, and sleep apnea).

Oxygen is transported from the surrounding environment to body tissue through two systems. The respiratory system moves oxygen from atmospheric air to the lungs (simultaneously moving carbon dioxide from the lungs to atmospheric air), and the cardiovascular system transports oxygen from the lungs throughout the body, where it is taken up by individual cells. Disease or failure within these two systems accounts for three out of the four top causes of death in the United States (similar to other developed countries, but see note below), which are heart disease, chronic lower respiratory diseases, and stroke (Murphy et al. 2013). When one considers that in the other top cause of death (cancer), the cardiovascular system is often locally affected by an increase in vasculature around the tumor site(s), it is clear that the accurate measurement of blood flow at various points in the body is crucial to the diagnosis and continued management of disease. (Note: It is very unfortunate that world mortality estimates are incomplete, especially since lower-income, less-developed countries tend to have the least complete reporting structures (Mathers et al. 2009). This section is in no way meant to ignore the devastating effects of other causes of death such as infectious and parasitic diseases, which most estimates rank as the second leading cause of death when considering the total world population.)

Historical Background

In 1950 a new ultrasonic method was introduced for measuring water velocity (Hess et al. 1950). This method used two transducers displaced from each other by a known distance and was inspired by interferometry, a common and precise technique of measuring relative translation or motion by utilizing spatiotemporal phase-change differences. As will be seen, this concept became central to most flow detection techniques from then through the decades until the present day. Similar devices were developed by other groups throughout the 1950s. One in particular (Kalmus 1954)

was designed to measure "slow" velocities (i.e., compared to those normally measured at the time in hydraulics) from 1 cm/s to 1 m/s with high accuracy. These results led that author to propose using the method to measure flow of liquid in a pipe, quickly leading several groups to propose similar methods for the purpose of measuring blood flow.

In 1957 this method was used to measure flowing blood (Baldes et al. 1957). In the same year, Satomura demonstrated the interpretation of Doppler-shifted ultrasound echoes between low frequency (heart wall) and high frequency (heart valves) (Satomura 1957). It is of particular interest to note that at this early time, he suggested the use of ultrasound for analysis of myocardial tissue stiffness, an area which has in more recent times grown significantly. By the end of the decade, at least two groups had demonstrated phase-difference ultrasound measurement for blood flow detection (Satomura 1959; Franklin et al. 1959). Although they represented a significant advance in cardiovascular medicine, there were two main disadvantages of these methods: they required two ultrasonic sensors (one for transmission and one for reception), and they necessitated invasive procedures, requiring direct contact with the vessel being interrogated. In 1964 the latter problem was solved with the development of a noninvasive, transcutaneous ultrasonic flowmeter (Baker et al. 1964).

In the meantime, pulse-echo ultrasound, a method by which a single transducer switches between transmission mode (briefly emitting sound) and receive mode (continuously receiving echoes reflecting from tissue), had been studied for the use of differentiating tissue types since at least 1949 (for a brief review, see Joyner and Reid 1963). Edler and Hertz appear to be the first to use this method in assessment of cardiac motion (Edler and Hertz 1954). Pulse-echo ultrasound equipment and systems designed for blood flow detection combined technologies gradually through the 1960s. In 1967, Baker and Watkins reported on a system that could simultaneously measure range and velocity with a single active element (Baker and Watkins 1967). Soon after, systems were developed that measured velocities at many ranges simultaneously by time gating the received signal at successive ranges (Wells 1969; Baker 1970). From this time forth, the use of ultrasound in cardiovascular medicine was firmly established. The stream of research articles in clinical journals covering cardiovascular ultrasound topics has grown steadily, branching out to cover topics in vascular supply to all major physiological systems.

This chapter starts with the physical principles of ultrasound and the use of blood flow detection by ultrasound. The main modes of blood flow detection are explained. Next, basic cardiovascular physiology is covered, especially the mechanisms that are responsible for continuous flow of blood. Several examples of disease and injury states of the cardiovascular system that are commonly detected using ultrasound are discussed. Finally, the chapter concludes with a brief look at two rapidly emerging ultrasound topics which are based on the same principles of tissue motion detection. Although they have both been studied for several years in the laboratory, they are increasingly appearing in the clinic and show promise of widespread acceptance for conventional use.

Physical Principles

All medical imaging systems produce a representation (visual, aural, or otherwise) of the interaction of energy and tissue. The energy-tissue interaction detected by an ultrasound imaging system is the reflection of mechanical (sound) waves from the body. It is interesting to note that ultrasound is the only major imaging modality to use solely mechanical energy; most other medical imaging systems detect either the transmission of electromagnetic energy through the body (e.g., X-ray, computed tomography) or emission of electromagnetic energy from the body (nuclear medicine, magnetic resonance imaging). In addition, the energy levels used in diagnostic ultrasound are nonionizing, making the modality very safe.

In ultrasound, mechanical energy is generated by electromechanical transducers, which convert electrical signals to mechanical waves through the piezoelectric effect. A simplified cutaway of a single-element transducer is shown in Fig. 1. Piezoelectric materials deform by compression or expansion when exposed to an electric field. Thus, an electrical signal at a certain frequency is applied to the electrodes of the ultrasound transducer to produce an oscillating mechanical deformation at the same frequency, generating a mechanical wave in the matching layer, which is then transmitted into the tissue. A very useful property of piezoelectricity is reciprocity: a piezoelectric material subjected to external mechanical forces will generate an electric field within the material. This property permits the use of the same transducer to generate a mechanical wave in the body and to "sense" mechanical waves from the body by creating an oscillating electrical signal in response to ultrasonic reflections. Currently, the most common piezoelectric materials used in ultrasound transducers are lead zirconate titanate (PZT) and polyvinylidene fluoride (PVDF).

As will be seen below, ultrasound imaging and ultrasound blood flow detection use the same "raw" data – that of pulse-echo reflections of mechanical energy from human tissue. This raw data is processed differently depending on whether it will be

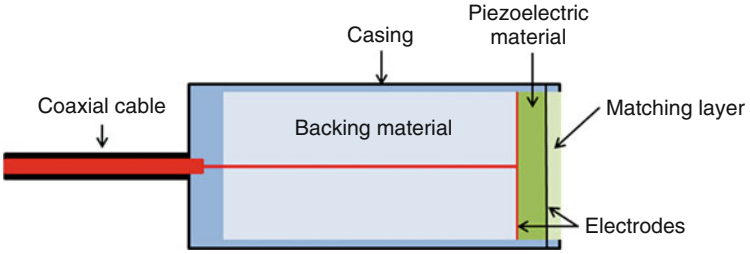

Fig. 1 Schematic (longitudinal cutaway) of ultrasonic transducer. A sinusoidal voltage pulse is applied to the electrodes, causing a change in shape of the piezoelectric material and generating a wave that travels through the matching layer and into the tissue being insonated. The backing material provides damping of the backwards-travelling part of the ultrasonic pulse (away from tissue) and has a significant effect on shaping the overall pulse. The matching layer has an acoustic impedance in-between that of the piezoelectric material and the tissue (specifically, the geometric mean), which maximizes transmission of energy into tissue

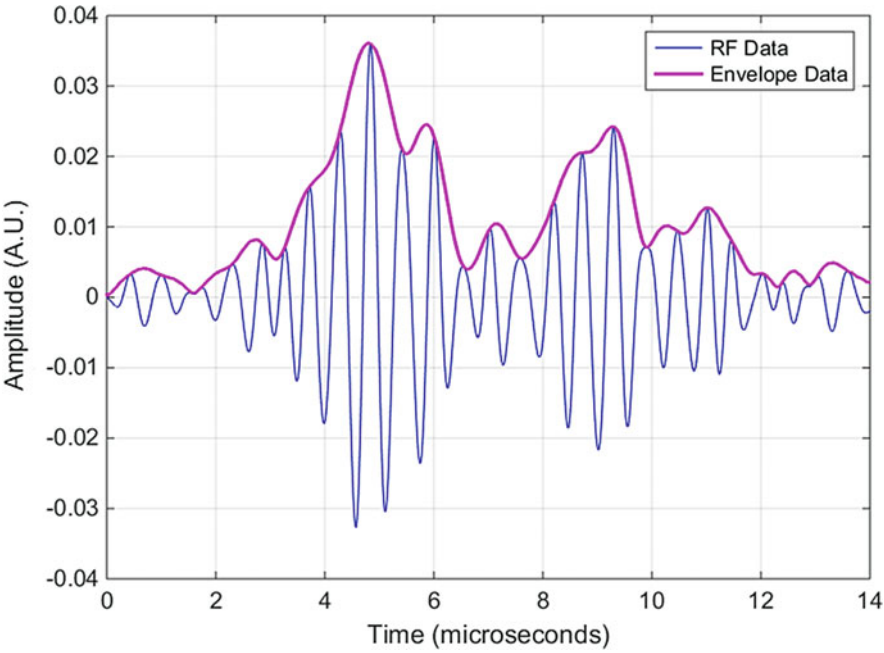

Fig. 2 Ultrasound A-line and envelope data. Typical appearance of digitally sampled ultrasound data from a single pulse-echo A-line. The data is sampled at 100 MHz (blue high-frequency curve, also known as radio frequency or "RF" data). The magenta curve overlaying the RF data is the envelope or "detected" data which is used in B-mode imaging

used for imaging or blood flow detection. A single pulse-echo reflection signal is termed an "A-line," where "A" stands for amplitude (Fig. 2). Briefly, in imaging, each A-line undergoes demodulation to extract the envelope, which is displayed on a screen with increasing echo amplitude portrayed as increased pixel intensity. Many A-lines are captured from different spatial locations, which together correspond to a two-dimensional plane intersecting the body. The collection of A-lines forms an image, known as a "B-mode" image where "B" stands for brightness. However, in blood flow detection, several A-lines are captured from the same point in space, with a phase change (described below) between A-lines then being used for blood flow estimation.

Although many algorithms have been studied over the last roughly 50 years to detect blood flow using ultrasound, the majority of commercial ultrasound machines today use three main modes. The first two (used more frequently) are known as "color flow" Doppler or "color Doppler imaging" (CDI), and "pulsed-wave" (PW) or "spectral" Doppler. The third mode, continuous-wave (CW) Doppler, is used somewhat less frequently. Although all of the techniques are termed "Doppler," only the third technique uses the true Doppler effect to detect blood flow. These three modes will be explained in more detail below. As will be seen, the particular physical setup of pulse-echo flow detection leads to flow estimate equations (used in the first

Table 1 Typical B-mode, color flow, and PW Doppler frequencies used in example applications

	B-mode (imaging)	Color flow	PW Doppler
Application	Frequency (MHz)	Frequency (MHz)	Frequency (MHz)
Abdomen	3.0–5.0	2.5–3.5	2.0–3.5
Breast	7.0–10.0	5.0–7.5	4.0–6.5
Fetal echo	3.0–5.5	2.0–5.5	2.0–3.5
Obstetrics	3.0–5.5	3.0–4.0	2.0–3.5
Digital	10.0–12.0	8.0–10.0	8.0–10.0
Musculoskeletal	7.0–12.0	5.0–9.0	5.5–9.0
Thyroid	6.0–10.0	5.0–7.5	4.0–6.0
Renal	2.5–3.5	2.0–3.0	2.0–3.0

two modes) that are very similar to the Doppler equation (used in the third mode), which can cause some confusion.

Typical ultrasound frequencies used clinically are from 2 to 12 MHz. Lower frequencies are able to penetrate deep into the body but have worse spatial resolution. Higher frequencies attenuate more quickly, and thus have less penetration in tissue, but have better spatial resolution and thus show more detail. Higher frequencies are used for structure close to the skin or where low attenuation is expected. Frequencies used for blood flow detection have a slightly narrower range than those used for imaging. Example applications with typical frequencies used for imaging, color flow, and PW Doppler are shown in Table 1.

Continuous-Wave Doppler

The Doppler effect as applied to blood flow detection occurs in a slightly different manner than in most "classical" derivations. Due to the transmit-receive nature of the system, and the fact that the ultrasound transducer does not move, there are actually two Doppler effects occurring when monitoring moving targets: the first happens when the stationary source (transducer) emits sound at frequency f_t (Hz or cycles/s) toward a moving object, which acts as an observer (red blood cells or moving tissue), leading to the blood observing a new frequency $f_t + f_{d1}$, where f_{d1} is a shift in frequency caused by the motion of the blood cells toward (or away from) the source. The second occurs when the red blood cells (now a moving source) reflect the frequency observed by the red blood cells back to a stationary observer, leading to the transducer observing a new frequency $f_t + f_{d1} + f_{d2}$. Consider the case where a single scatterer (perhaps a single red blood cell) moves at velocity v relative to the stationary transducer (Fig. 3a), where positive v (m/s) is movement toward the transducer. When a mechanical (sound) wave is emitted from the transducer and transmitted to the position of the moving scatterer, the spatial wavelength of the

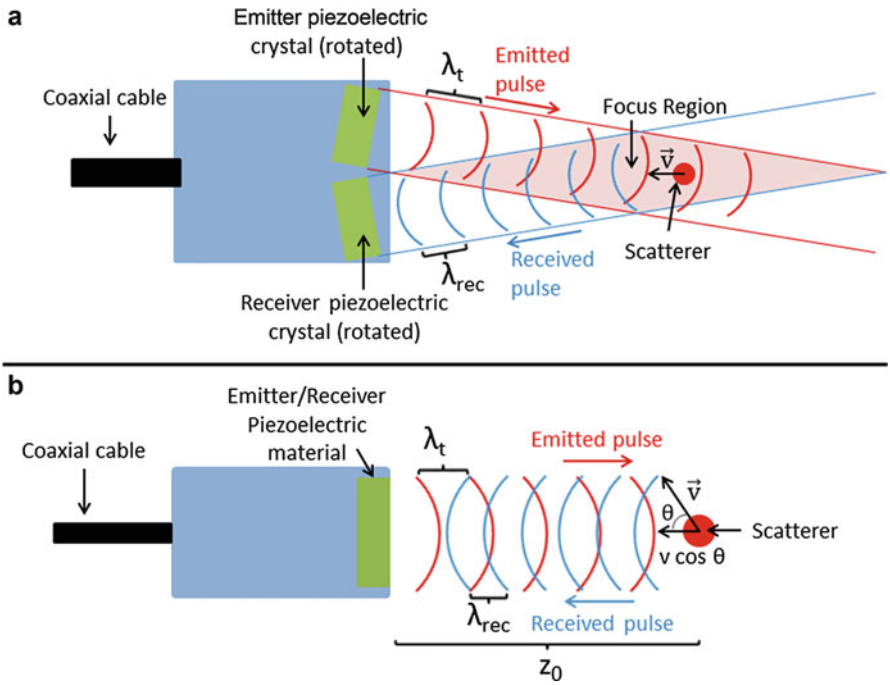

Fig. 3 Operation of continuous-wave (CW) and pulsed-wave (PW) Doppler transducers. *Top* (**a**), Operation of a continuous-wave Doppler transducer. A continuous wave is emitted by one piezoelectric crystal within the transducer and is reflected by scatterers in the focus region. A second piezoelectric crystal within the transducer receives the reflected signal, which has a wavelength λ_{rec} (m) that is either less than or greater than the emitted wavelength λ_t (m), depending on whether the velocity v (m/s) of the scatterer is directed towards or away from the transducer. *Bottom* (**b**), Operation of a pulsed-wave Doppler transducer. A pulse is emitted from the transducer, is reflected by a moving scatterer in the blood at a distance z_0 (m) away from the transducer, and then received by the transducer and recorded. The wavelength of the received pulse λ_{rec} (m) will be less than or greater than the wavelength of the emitted pulse λ_t (m), depending on whether the velocity v (m/s) of the scatterer is directed towards or away from the transducer

mechanical wave observed by the moving scatterer is different than that observed by a stationary scatterer. If the spatial wavelength observed by a stationary scatterer is λ_t(m), the wavelength observed by a moving scatterer will be shifted by an amount equal to the distance the scatterer has traveled between emission of wave peaks from the source, which is vT_t(m), where T_t is the time period (or wave period) for one complete cycle $(= 1/f_t)$. For waves, the speed of sound c is related to the frequency and wavelength by $c = \lambda f$. The wave period T_r observed by the scatterer is therefore

$$T_r = T_t - \frac{vT_t}{c} = \left(1 - \frac{v}{c}\right)T_t \tag{1}$$

where c is the speed of sound in m/s. The perceived temporal frequency by the scatterer is

$$f_r = \frac{1}{T_r} = \frac{1}{T_t(1 - v/c)} = \frac{f_t}{(1 - v/c)} \tag{2}$$

thus, the one-way Doppler shift frequency f_{d1} is the difference between the received temporal frequency and the transmit frequency:

$$f_{d1} = f_r - f_t = f_t\left(\frac{1}{1 - v/c} - 1\right) = f_t\left(\frac{v}{c - v}\right) \tag{3}$$

For ultrasound blood flow measurement, the nominal c value for soft tissue is 1,540 m/s, and blood flow velocities are normally much less than 1 m/s (except for partially obstructed vessels, for which the blood flow velocity can be a few m/s). In all cases, $c >> v$, and the denominator of Eq. 3 is thus about c:

$$f_{d1} = \frac{v}{c} f_t \tag{4}$$

showing that a scatterer moving toward the source will observe a positive Doppler shift (an increase in frequency) and vice versa. On reflection, the same reasoning applies, except that the new "source" frequency emitted (reflected) by the scatterer is f_r from Eq. 2. A doubling of the Doppler shift frequency ensues, and thus the return frequency shift (two-way transmit-receive Doppler shift) observed by the transducer is

$$f_{d2} = \frac{2v}{c} f_t \tag{5}$$

If the direction of the scatterer's motion is not directly toward or away from the transducer, the above reasoning applies for the component of the velocity vector in the direction of the acoustic beam, which is $v \cos \theta$ (see Fig. 3b). Thus, the final Doppler shift seen by the transducer is

$$f_d = \frac{2v \cos\theta}{c} f_t \tag{6}$$

It is interesting to consider the typical range of Doppler shift frequencies that occur in ultrasound. Note that Eq. 6 shows that the Doppler shift is linearly proportional to the ratio of blood or tissue velocity and speed of sound. Common blood velocities in vessels interrogated with ultrasound range from tens of cm/s to hundreds of cm/s. (Wider ranges do exist; blood flow is very slow in capillaries, can be near zero at the vessel intima-lumen interface, and can be several m/s just distal to obstructed vessels.) The nominal value of c used by commercial machines is 1,540 m/s, and thus the typical range of (v/c) is 10^{-2}–10^{-3}, making Doppler shifts two to three orders of magnitude below the transmit frequency. With typical transmit

frequencies of 2–10 MHz, typical Doppler shift frequencies are in the low to mid kHz range, which is in the audible frequency range. Therefore, the Doppler shift frequency detected by ultrasound is commonly sent to a speaker, which gives an aural representation of the blood flow velocities detected. The faster the blood flow velocity detected, the higher the frequency (pitch) is heard from the speaker. Physicians trained in Doppler blood flow have learned to identify a wide range of blood flow conditions, both normal and pathologic, by listening to this sound. This sound is not the sound that would be produced by a hypothetical microphone within the body near a blood vessel; it is an aural "translation" of the blood flow velocities into sound.

Continuous-wave Doppler requires two transducers (in contrast to pulse-echo Doppler described below), one for continual emission of sound and one for continuous reception of echoes. This blood flow detection configuration is the only one to make use of an actual Doppler frequency shift as shown in Eq. 6. The main advantages of continuous-wave Doppler are high sensitivity to flow anywhere along the beam axis and no possibility of aliasing (described below), which enables unambiguous detection of high-velocity flow. The main disadvantage is lack of depth information, i.e., how deep into the body the blood flow is detected. Common uses of continuous-wave Doppler ultrasound are fetal heart monitoring during early pregnancy, labor, and delivery, deep venous studies, and flow measurement across heart valves with suspected stenoses.

Pulse-Echo Doppler

Most ultrasound blood flow examinations use a pulse-echo velocity estimation scheme requiring only a single transducer for both transmission and reception of sound waves. Pulse-echo Doppler has two variations. The first is termed spectral Doppler, in which a large number of (typically 64–128) pulse-echo signals are used to obtain information about the distribution of blood flow velocities in a small area of the body. The second is termed color Doppler, in which fewer pulse-echo signals (typically 6–16) are used to quickly estimate a single parameter, the mean velocity (via the mean frequency shift) of the velocity distribution over a larger area of the body (at many locations) in a short time span allowing the display of this information at "real-time" frame rates (typically 10–30 frames/second). The two methods are complementary: spectral Doppler provides detailed spectral information (i.e., the distribution of blood flow velocities) in a small spatial area (typically within a blood vessel lumen), while color Doppler provides less information (mean velocity) over a large field of view. Often, both modes are shown together and are called either "duplex" mode (the dual combination of color flow and PW Doppler) or "triplex" mode (the triple combination of B-mode, color flow, and PW Doppler). The terms have become almost synonymous since color flow is rarely if ever shown without the corresponding B-mode image underneath. In this chapter, the combined modes will be referred to as "triplex." Figure 4 shows an example of a carotid artery in triplex

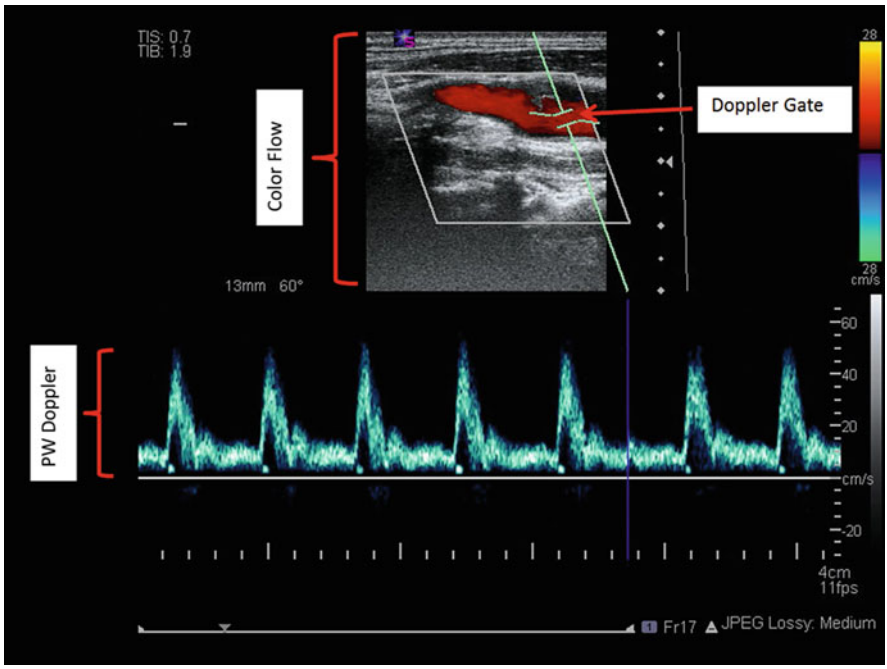

Fig. 4 Example of triplex mode. Common carotid artery, showing mixture of high resistance flow due to the downstream high resistance of the external carotid artery and low resistance flow due to the downstream low resistance of the internal carotid artery (flow is from *right* to *left* in the image). Note the superposition of color flow on top of the B-mode image, with a PW Doppler spectrum underneath

mode. The mean velocity across the carotid (color flow) can be seen in the top half of the figure, while detailed velocity spectra vs. time (PW Doppler) are seen in the lower half of the figure.

Although neither method uses the classical Doppler effect, they are often termed "Doppler" because the physical principles leading to a "Doppler" signal result in essentially the same mathematical expressions as for true Doppler. Both methods rely on the change in phase at a certain distance in pulse-echo measurements when ultrasound scatterers are in motion. An idealized example may help make this clear. Assume a transducer is pulsed such that it emits a sinusoidal "burst" of several cycles toward a scatterer moving with velocity v and located at a distance z_0 away from the transducer (Fig. 3b). The component of the scatterer's velocity in the direction toward or away from the transducer is given by $v\cos\theta$. Therefore, if the pulse was sent at time t=0 s, the pulse returns to the transducer at time $t_0 = 2z_0/c$. Now suppose another pulse is sent toward the scatterer after a delay time of ΔT(s). At the time the second pulse encounters the scatterer, the scatterer will have moved relative to the transducer to a new position

$$z_{new} = z_0 + \Delta z = z_0 + v(\Delta T)\cos\theta \tag{7}$$

and thus the second pulse returns to the transducer at time

$$t_{new} = \frac{2z_{new}}{c} = \frac{2(z_0 + v(\Delta T)\cos\theta)}{c} \tag{8}$$

An assumption is made that the scatterer reflects the pulse ideally, i.e., the pulse shape returning to the transducer is the same shape as the emitted pulse. Note that this assumption actually *neglects* the true Doppler effect, i.e., the return pulse is assumed to have the same frequency content as the emitted pulse. In practice, this process is repeated a number of times. Each pulse is emitted at carefully controlled time intervals ΔT, and each pulse-echo is sampled at a time corresponding to a constant depth. The inverse of ΔT is known as the pulse repetition frequency (PRF). The left side of Fig. 5 shows a diagram of a succession of A-lines, demonstrating the collection of a sample from each one at the same pulse-echo time. Between each pulse, the scatterer has moved to a new location, and the pulse will therefore return to the transducer at a different time (in this case, later and later since the scatterer is moving away from the transducer). The right side of Fig. 5 shows the samples on a graph where each unit is a multiple of ΔT. Notice that since the scatterer is moving at a constant velocity, the shift in the location on the pulse that is sampled from one pulse-echo to the next is the same. In this idealized scenario, we are simply re-sampling the original pulse. This results in the graphing of another sinusoidal burst, but on a new axis with a different time scale. The conversion to "Doppler" comes by considering what the frequency of this new sinusoid is. A sinusoid is characterized by its frequency, which is the derivative (time rate) of change in phase:

$$\omega = \frac{d\phi}{dt} \tag{9}$$

The change in phase detected between pulse-echo signals is related to the change in scatterer distance by the wavenumber $k(\text{radians/m}) = 2\pi/\lambda$. From Eq. 7, the change in scatterer distance between pulses is $\Delta z = v\Delta T\cos\theta$. The change in distance traveled by the interrogating pulse is twice this amount, since it travels to and from the scatterer. The corresponding change in phase Δϕ is

$$\Delta\phi = k2v(\Delta T)\cos\theta = \frac{2\pi}{\lambda}2v(\Delta T)\cos\theta = \frac{2\pi f_t}{c}2v(\Delta T)\cos\theta \tag{10}$$

Therefore, the change in phase per unit time, or frequency, is

$$\frac{\Delta\phi}{\Delta T} = 2\pi\frac{2v\cos\theta}{c}f_t \tag{11}$$

in radians/s. Notice that the original frequency f_t has been simply *scaled* by the component of the velocity in the direction of the transducer divided by the speed of

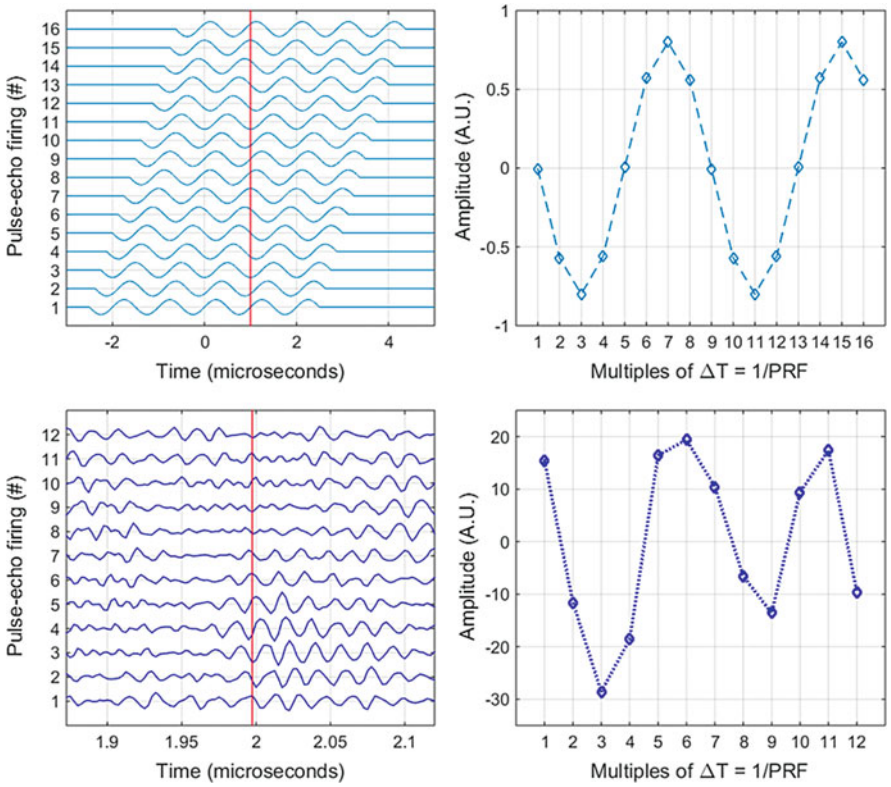

Fig. 5 Idealized and actual pulse-echo data examples. *Top left*, 16 idealized successive pulse-echo A-lines shown on a constant time base from *bottom* to *top*. The vertical *red line* represents the constant time or depth at which each A-line is sampled. *Top right*, the 16 samples acquired from each of the idealized A-lines plotted on a new graph, scaled by the inverse of the pulse repetition frequency. The resulting signal is a sampled version of each A-line. *Bottom left*, 12 successive actual pulse-echo A-lines from a common carotid artery shown on a constant time base from *bottom* to *top*. The vertical *dashed line* represents the constant time or depth at which each A-line is sampled. *Bottom right*, the 12 data samples acquired from each of the A-lines plotted on a new graph, scaled by the inverse of the pulse repetition frequency. The frequency spectrum of the resulting signal carries velocity information

sound. This factor is the same as that in continuous-wave Doppler. The "Doppler" frequency f_d in units of Hz or cycles/s is

$$f_d = \frac{2v\cos\theta}{c} f_t \tag{12}$$

which is the same as Eq. 6. It can be helpful to think of this "Doppler" frequency as a scaling of the transmitted frequency rather than a physical shift in the frequency.

If the applied pulse is not a pure sinusoid burst, but a signal containing a range of frequencies, then it can still be assumed that the signal shape does not change between firings. If the pulse-echo reflection "system" is assumed to be linear, then again the result is a sampled version of the original pulse. The Doppler signal looks like the applied pulse, just on a different time scale. Every frequency present in the wideband pulse is scaled by the ratio of the blood velocity to the speed of sound.

Several factors contribute to keep this idealized situation from occurring in vivo. For example, there are multiple scatterers, the beam shape is not uniform, and scatterers move in and out of the beam during flow data collection. Collectively these factors cause the signal to decorrelate, and in reality successive A-lines gradually stop "looking like each other," and thus the final Doppler signal looks like none of the original A-lines. However, the phase relationships between adjacent firings remain, to the first order, directly related to velocity, which is essential for accuracy of the flow estimation algorithms. The bottom two graphs of Fig. 5 show actual data taken from a carotid artery. Note how on the lower left side of Fig. 5, pulse-echo firings which are adjacent to each other are more alike, but those farther away lose their resemblance.

The primary disadvantage of Doppler-based methods is that they are only able to detect velocity components parallel to the direction of the ultrasound beam. If the blood flow is not parallel to the ultrasound beam, only the vector component of flow along the direction of the beam is detected. Velocity measurements are often angle corrected, but this requires manual rotation of a cursor. If the angle correction is inaccurate, large errors in velocity estimation may occur (Wells 1998). In order to address this problem, several estimation algorithms different from Doppler have been proposed that include information about flow in the lateral (and even azimuthal) direction perpendicular to the axial direction. Some of the more prominent examples of multidimensional flow estimation are given in the "Potential Applications" section below.

A limit to Doppler-based methods of flow detection is the Nyquist criterion, which states that the maximum Doppler frequency must be less than or equal to one half of the pulse repetition frequency to avoid aliasing. Thus, aliasing limits the maximum detectable velocity of blood flow. This is usually not a major problem clinically, as (1) aliasing is normally apparent because of the color pattern or spectral cutoff of the peaks and thus the operator can visually see it occurring, and (2) the PRF in most cases can be adjusted to meet this criterion. In the cases where the maximum PRF of the machine (at the particular depth) is not high enough to avoid aliasing, it is usually because of abnormally high blood flow velocities such as those just distal to a stenosis, and the diagnosis can be made through the combination of an abnormal flow pattern and the knowledge that aliasing is occurring.

In either color flow or PW Doppler, the ultrasound machine ends up with a discrete-time signal representing the samples taken at fixed times from successive pulse-echo firings (i.e., the right graphs in Fig. 5). In both modes, the object is to estimate the frequency content of this discrete-time signal. In color flow, where only a few (typically 6–16) samples are present in the signal, the system developed by

Kasai (Kasai et al. 1985), which uses two values of the complex autocorrelation of the discrete-time signal, is still used. This algorithm returns one value, an estimate of the mean frequency of the spectrum of the signal. In PW Doppler, many more samples are taken (typically 64–128) and the entire spectrum is analyzed, usually with a fast Fourier transform (FFT) or a similar variant. Spectral estimation is a well-studied topic, and many techniques have been studied for use in blood flow velocity measurement (David et al. 1991).

Basic Cardiovascular Physiology

The cardiovascular system, from a biomedical engineering point of view, is a highly regulated network of closed channels (arteries, veins, and capillaries) with a driving force (heart) that transports blood throughout the body. In general, the oxygen carried in vessels is directly proportional to the volume of blood therein. Therefore, regulating the amount of blood that flows to a particular organ or physiological system is equivalent to regulating the oxygen delivered to that system.

Many factors are involved in the overall feedback mechanisms that regulate blood flow to different parts of the body. Oxygen lack in tissue is sensed by remarkably complex networks via a number of pathways on the cellular and subcellular levels (Giaccia et al. 2004). However, there are few ways to physically *change* the amount of blood flowing to a particular place. Short-term (on the order of a few seconds) compensatory physical effects include the widening (vasodilation) and narrowing (vasoconstriction) of blood vessels, increased heart rate, and increased cardiac pumping pressure. Longer-term effects (on the order of days to years) include increased production of erythrocytes to increase the amount of oxygen held per volume measure of blood and thickening of the heart muscle. These effects may be healthy or normal (such as vasodilation/vasoconstriction during normal everyday activities), and some may be pathological (e.g., left ventricular hypertrophy, which is an abnormal thickening of the heart wall in response to an increased workload, decreasing the efficiency of the heart).

Physiologists divide the cardiovascular system into two subsystems or "circuits." The pulmonary circulation moves blood that is partially depleted of oxygen from the right side of the heart to the lungs, where it is oxygenated and then back to the left side of the heart. The systemic circulation moves oxygenated blood from the left side of the heart to the rest of the body, eventually moving through small blood vessels (capillaries) in different physiological systems. Oxygen transfer takes place across the capillary walls by cells adjacent to the capillaries, partially depleting the blood of oxygen, which then moves back to the right side of the heart. In a simplified sense, the systemic circulation can be thought of as an energy source (the pumping action of the heart) causing blood to flow through parallel branches (each branch being an organ or a physiological system). Each of these branches has an associated resistance, which governs the volume flow rate of blood in that branch. The

resistance in each branch is dynamically altered by the regulatory mechanisms described above.

The physical laws that govern flow through a blood vessel describe the relationships between fluid pressure, vessel resistance, and vessel size. Volume flow of blood (Q, ml/min) is directly proportional to the change in pressure between two points in a vessel (ΔP, mmHg or Pa) and inversely proportional to vessel resistance (R):

$$Q \propto \frac{\Delta P}{R} \tag{13}$$

The resistance to flow is directly proportional to the vessel length L (between the two points across which the change in pressure exists) and the viscosity η and inversely proportional to the *fourth* power of the vessel lumen radius r:

$$R \propto \frac{L\eta}{r^4} \tag{14}$$

It is this strong dependence on the lumen radius that permits a wide range of flow rates with relatively small vasoconstriction or vasodilation. The vessel length and blood viscosity do not change significantly in healthy humans, so the primary regulation of blood flow is driven by the change in pressure and the resistance. Combining (13) and (14) with proper physical constants gives Poiseuille's law:

$$Q = \frac{(\Delta P)\pi r^4}{8L\eta} \tag{15}$$

These factors have a noticeable effect on the Doppler modes. For example, the common carotid artery shown in Fig. 4 splits into the external carotid artery (Fig. 6, top), supplying a high-resistance network of relatively smaller vessels in the outer neck and facial tissues, and the internal carotid artery (Fig. 6, lower), which supplies a low-resistance network of relatively larger vessels into the brain. The PW spectra are typical of high-resistance and low-resistance flow, respectively (while Fig. 4 is a combination of the two). The regulation of vessel radius (vasoconstriction/vasodilation) can happen very quickly. Figure 7 shows an "internal" view of what happens during blood pressure measurement via cuff inflation over the brachial artery. In Fig. 7 (top), the cuff is quickly inflated in a few seconds from zero pressure to a pressure exceeding the systolic pressure, temporarily preventing flow in the artery. In Fig. 7 (bottom), the cuff is shown in rapid deflation. The artery responds to the downstream oxygen loss by temporarily vasodilating and exhibiting a low resistance flow pattern. Within 20 heart cycles, the Doppler spectrum appears normal again (not shown). (This demonstration is not quite the same since during blood pressure measurement, the cuff is slowly deflated until pressure drops below its end diastolic value.)

Flow patterns are also affected by the location of the vessel in the body, the vessel health, and the positioning of the ultrasound transducer. Two more examples

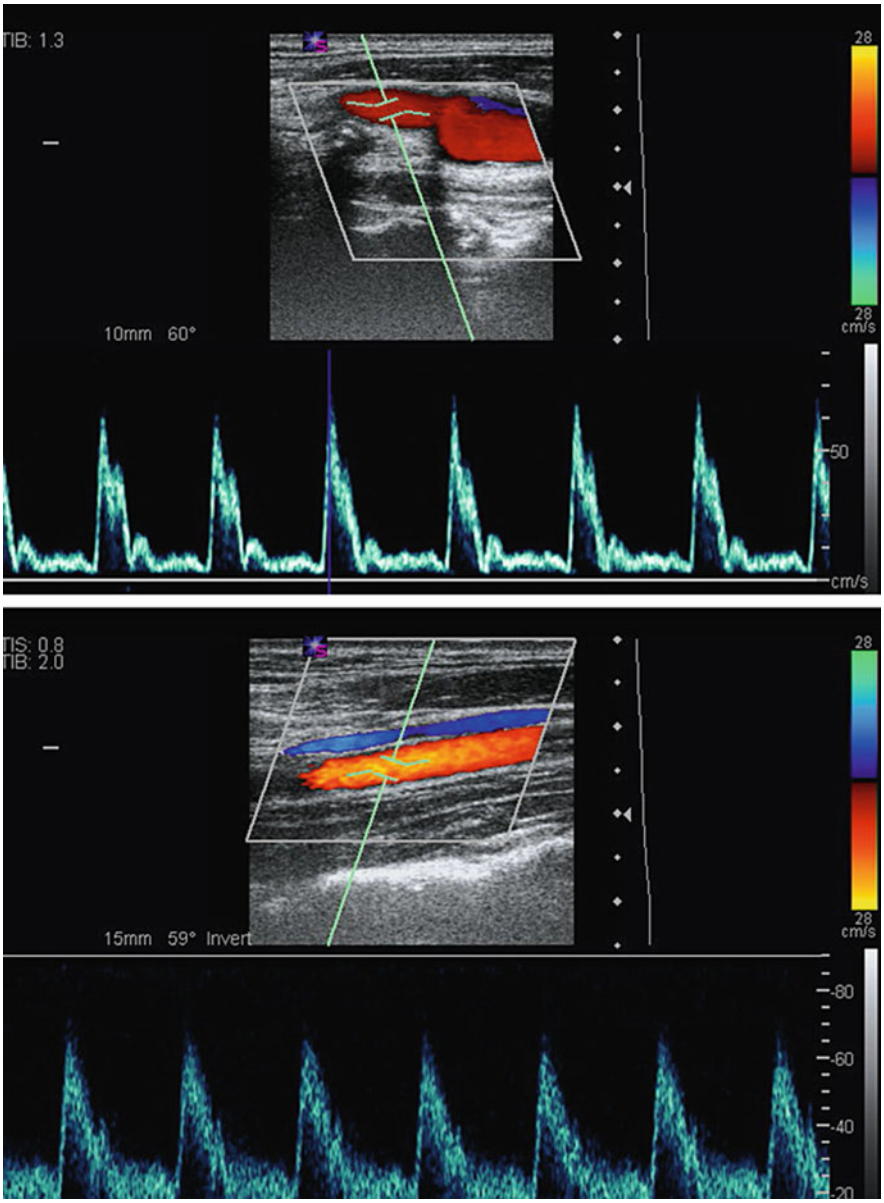

Fig. 6 External and internal carotid arteries. *Top*, longitudinal view of external carotid artery (ECA), showing high resistance flow. The Doppler spectrum is narrow (i.e., at any given time, few velocities exist inside the Doppler gate). Bottom, longitudinal view of internal carotid artery (ICA), showing low resistance flow. At each point in time, a broader range of velocities is present within the Doppler gate relative to high-resistance flow. Opposite flow (*blue*) in the jugular vein can be seen above the ICA in color flow. (Note: "invert" mode is being used on the ICA PW spectrum due to the chosen angle of the Doppler line; negative flow (*red* on the color map) is from right to left in the ICA color flow image; for sake of clarity invert mode is sometimes used to avoid viewing the spectrum "upside down"

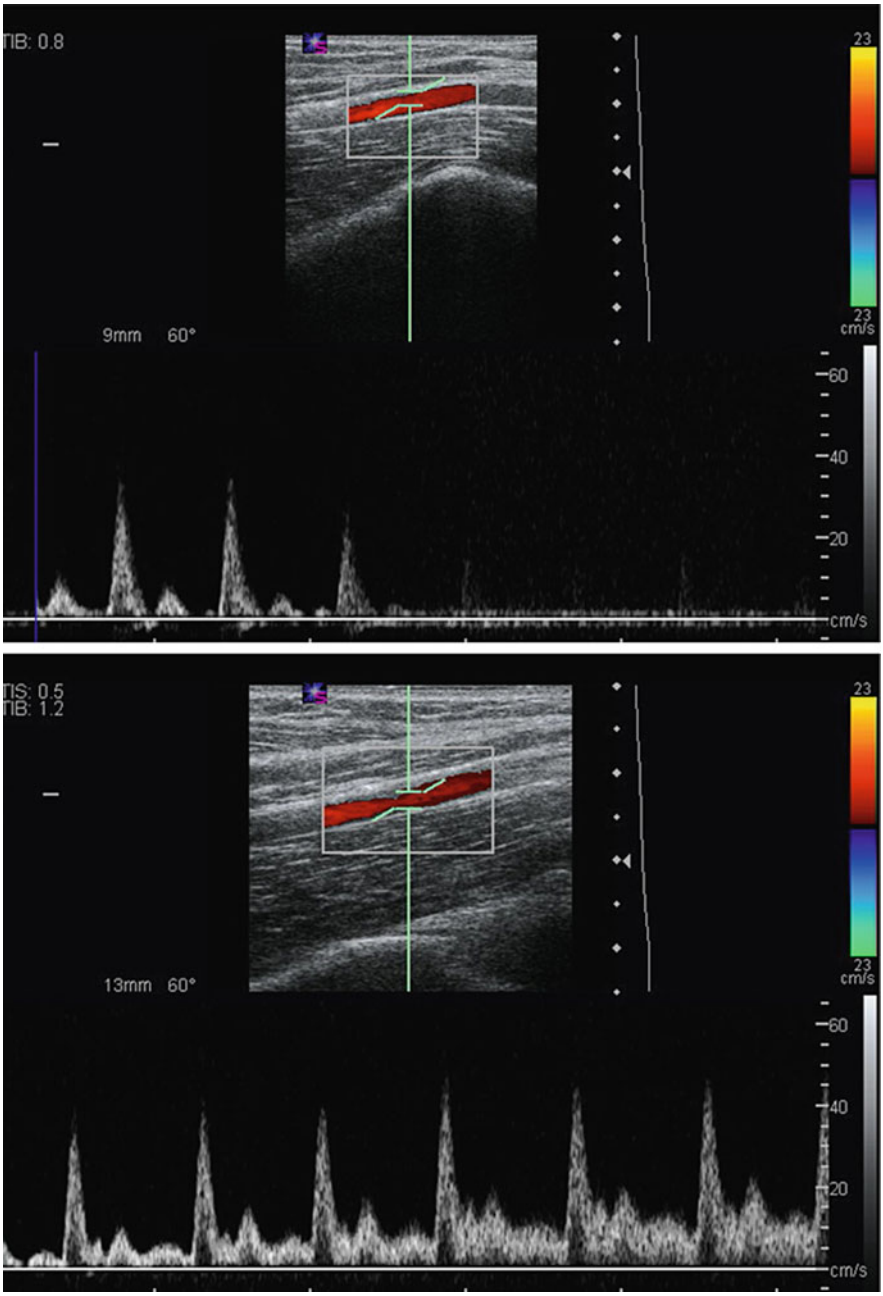

Fig. 7 Brachial artery during pressure cuff inflation and deflation. *Top*, brachial artery shown during pressure cuff inflation, representing five seconds of time. Note the decreasing amplitude of arterial velocity in the PW Doppler spectrum as the cuff gradually surpasses the systolic pressure and flow is cut off. (The color flow view is static, representing the view seen at the beginning or left side of the PW Doppler display). The Doppler angle was not corrected past 60°, so velocities are

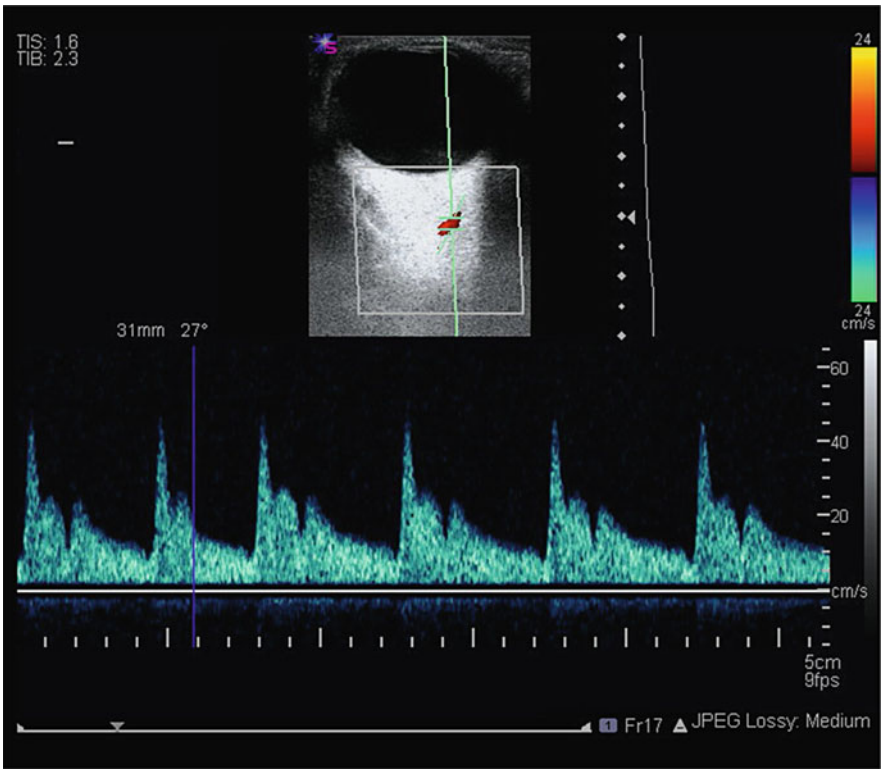

Fig. 8 Ophthalmic artery. Triplex mode view of ophthalmic artery. Note that only a small segment is visible in the color image. This is because the ophthalmic artery in most humans courses such that it does not stay in any one plane for a long length. The *large dark circle* above the color box is the interior of the eye

demonstrate the applicability of flow measurement throughout the body, and the wide range of flow patterns that may be seen even in normal patients. Figure 8 shows flow measurement in the ophthalmic artery, which lies behind the eye. Figure 9 shows flow measurement in the femoral artery, taken in the upper leg.

It is important to note that conventional ultrasound Doppler algorithms measure blood *velocity* (cm/s) and not blood *flow* (ml/min). In addition, only the vector component of flow along the direction of the ultrasound pulse can be measured. Thus, a single velocity vector measurement includes the velocity amplitude and

Fig. 7 (continued) actually higher than shown. *Bottom*, brachial artery during pressure cuff deflation, also 5 s of time. Note the sudden onset of decreased resistance flow, indicated by the broader Doppler spectrum, just after pressure cuff fully deflates (approximately midway or 3 s across the screen)

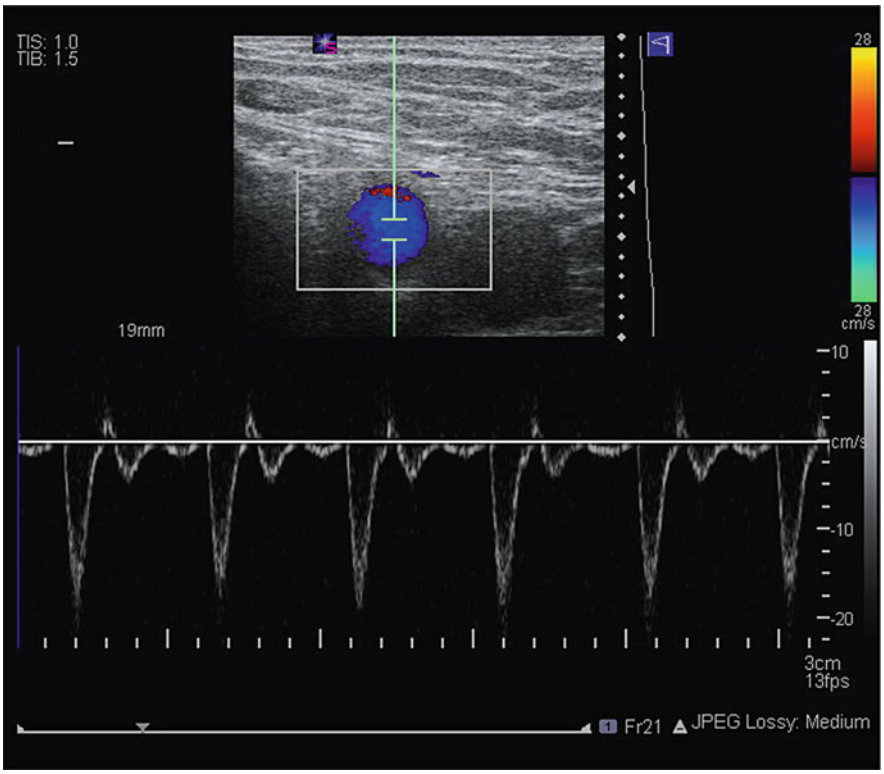

Fig. 9 Transverse view of femoral artery and vein. The femoral artery and vein in triplex mode. Both artery and vein are in transverse view, making them appear as dark circles. The Doppler gate is positioned over the artery, with the larger-diameter vein just to the right. Even in transverse view, a weak Doppler signal can be detected in the artery due to the three-dimensional nature of the ultrasound beam. Note the triphasic nature of the flow

whether the flow is toward the transducer or away from it. If the flow rate is desired, there are a limited number of ways to estimate the volume flow rate given the vessel diameter (which sometimes may be measurable in the ultrasound image) and the velocity profile across the lumen (requiring multiple flow measurements at different depths). However, to date, the majority of cardiovascular studies using ultrasound rely on velocity measurement to assess function and pathology. Over a period of about 40 years, a broad clinical knowledge base has been built up using the velocity and/or the velocity distribution. Many pathological conditions can be determined from comparing the velocity measurements (usually over time) in a patient with known normal patterns. Furthermore, the velocity distribution at a point in the body may indicate a problem elsewhere; in the circuitous nature of the cardiovascular system, a pathological condition at one point in the circuit can cause upstream or downstream effects at another point.

Cardiovascular Assessment

Cardiac Pathologies

The Doppler and phase-shift detection algorithms discussed above may be used to estimate motion in all tissues that reflect a strong enough ultrasound echo to allow detection of frequency or phase changes. Blood is actually classified as a connective tissue because of the large amount of extracellular material (notably plasma) that exists within it. In echocardiography, "motion estimation" may refer to blood flow detection, cardiac muscle tracking, or vessel wall movement. The latter two are sometimes referred to as Doppler tissue imaging, or DTI. As such, it is appropriate to briefly mention applications of Doppler ultrasound within the heart itself.

The main cardiac pathologies for which ultrasound tissue and blood motion detection are useful include abnormal size, especially of the left ventricle (Patil and Wiegers 2014), valvular defects and diseases (Zeng et al. 2014; Nishimura et al. 2014), and prenatal screening for congenital heart disease (Hunter and Simpson 2014). Stenoses of the great vessels are covered below in vascular pathologies. Of course, ultrasound *imaging* (i.e., B-mode) is also used in many cardiac structural assessments and is a primary tool in cardiology; the purpose of this section is to briefly review blood flow and tissue motion applications.

Cardiomyopathy is a condition that affects the pumping ability of the heart due to abnormal dilation of the left ventricle, thickening of the muscle wall, or increased stiffness of the muscle. Doppler tissue imaging and its variants show relative movement of the heart chamber walls and valves. Wall motion assessment is important because abnormal heart motion may be detected before associated loss in volume flow out of the left ventricle (Lee 2004). Similar analyses can be performed in fetuses or infants with congenital heart disease. These children often have abnormal ventricular geometry and load conditions on their hearts during different phases of systole and diastole. This results in a difference in tissue velocity, which can be measured and compared with reference values from healthy children (Eidem 2009).

In addition to stenosis, valvular heart disease often leads to the inability of one-way valves within the heart or acting as gateways out of the heart to completely seal. Incomplete sealing leads to a backflow of blood, known as regurgitation, in which the pressure difference across the valve forces blood backward through the valve. Valves possibly affected include the aortic (between left ventricle and aorta), mitral (between left atrium and left ventricle), pulmonary (between right ventricle and pulmonary artery), and tricuspid (between right atrium and right ventricle). Regurgitation can be seen in color flow by a thin jet of flow in the reverse direction than expected, across the valve during the phase of the heart cycle when the valve is closed and pressure is building past the valve.

Vascular Pathologies

The main vascular pathologies for which ultrasound blood flow detection is useful include vessel narrowing (stenosis), occlusions or blockages, vessel wall

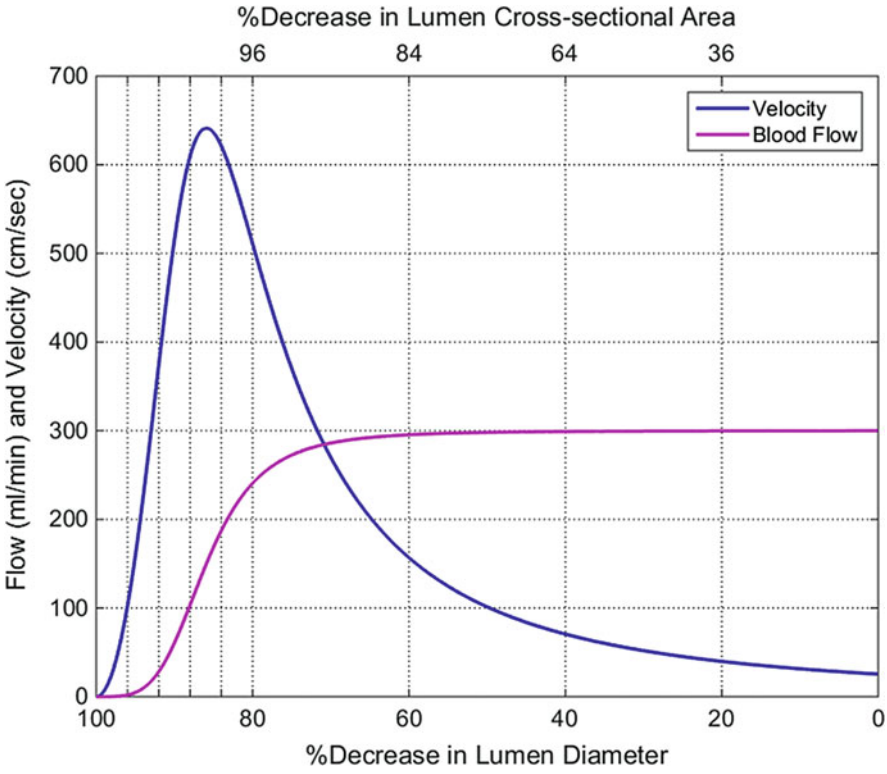

Fig. 10 Spencer's curve. Re-creation of Spencer's curve from equations and example blood vessel values given in Spencer and Reid (1979). Here, unobstructed lumen diameter = 5 mm, stenotic length = 2 mm, viscosity = 0.04 Poise, and nominal flow rate = 300 ml/min

abnormalities, abnormal vessel development, and emboli or thrombi in the blood. Along with imaging, assessment of the Doppler spectrum, especially the degree of resistance and/or turbulence indicated, is the primary ultrasound diagnostic tool.

Stenoses in the blood vessels can arise from a variety of sources, e.g., atherosclerotic disease, thrombi, and physical trauma. Spencer and Reid developed a model using the flow equations above and the biophysical principle of continuity of flow in a closed system (Spencer and Reid 1979). This model (recreated in Fig. 10) shows both the flow rate and velocity changes with respect to the percent change in narrowing in a vessel containing an axisymmetric narrowing. Although the model was developed with certain assumptions (e.g., straight walls, no bifurcations, no compensatory downstream vasodilation), it has been used extensively in studies of interpretation of Doppler spectra, especially in cerebrovascular ultrasound (Alexandrov 2007). Most notably, with progressive obstruction (moving right to left in Fig. 10), it is seen that velocity starts to increase significantly before the flow rate significantly decreases. Thus, since the flow rate is the parameter most tied to oxygen delivery, vessel narrowing can be inferred from the Doppler spectrum before injury to downstream tissue occurs. Of course, many other factors affect this

relationship, and vascular laboratories commonly standardize their own protocols using several measurements of blood flow velocity in different locations around the stenosis (Grant and Melany 2012).

The degree to which Doppler spectra reflect progressive stenosis toward occlusion also depends on the location of the problem. For example, atherosclerosis is a disease resulting from accumulation of plaque on the inner arterial wall. Around the heart, atherosclerosis in the coronary arteries can lead to a heart attack; in the arms and legs, atherosclerosis can lead to peripheral arterial disease (PAD) resulting in insufficient blood flow to the extremities; atherosclerosis in the carotid artery (which supplies the brain) can lead to stroke. Stenosed arteries closer to the heart exhibit recognizable flow disturbance patterns in the Doppler spectra. Such disturbances typically include abnormally high velocities at the obstruction site and development of turbulence just distal to the stenosis, resulting in spectral broadening (change from a narrow range of velocities at peak systole to a wider range of velocities) and even reversal of flow (eddy formation near the walls of the vessel due to the transition from the narrowed opening back to a region of normal diameter) (Douville et al. 1985). However, note the very rapid change of velocities in the last few percent of decreased cross-sectional area in Fig. 10 (the transition from "near occlusion" to "total occlusion"). In such severe stenoses, peak systolic velocities may be significantly decreased and diastolic velocities may disappear in the Doppler spectrum. With a total occlusion, flow will not be sensed within the blocked vessel; however, if the blocked vessel is an artery, low but equal amounts of forward and backward flow may be seen in the spectra resulting in no net flow.

Evidence of a blockage in one vessel can sometimes be seen in the spectra of another vessel. A classic example is subclavian steal syndrome. The subclavian arteries (paired, right and left side) are larger arteries branching off from close to the heart; they supply the arms. From each subclavian artery, a vertebral artery branches off, slightly smaller in size, and supplies the posterior (back) side of the brain. If the subclavian artery is normal, flow seen in the corresponding vertebral artery's Doppler spectrum is typically in one direction (toward the brain). An occlusion in the subclavian artery can cause either the presence of both forward and reversed flow in the vertebral artery (incomplete steal) or completely reversed flow away from the brain and toward the branching point (complete steal). In essence, the body "steals" blood from the posterior cerebral circulation in order to supply the arm.

Vessel Wall Physical Problems

Atherosclerosis may also be secondary to many other disorders, such as diabetes, familial hypercholesterolemia, growth hormone deficiency, and end-stage renal disease (Lehman 1993; Sahin 2013). Before accumulation of plaque causes dangerous narrowing of the vessel, the mechanical properties of the blood vessel change, particularly the elastic modulus or stiffness of the walls. It is possible to detect this property change using ultrasound. Thus, measurement of arterial stiffness may provide an early predictor of cardiovascular risk.

Degeneration or weakening of the vessel wall can lead to an aneurysm, which is an abnormal widening (a "bulge" or "ballooning") in the vessel. Aneurysms

containing all wall layers ("true" aneurysms) exhibit lower local flow resistance due to the transient wider opening. Doppler spectra typically show lower velocities and a lower-resistance waveform in general. A "dissecting" aneurysm occurs when a tear in the inner wall develops, causing a pocket to form between two wall layers of the vessel. Blood will flow into this pocket and can often be seen in color flow as a reversed-flow component within the vessel. A pseudoaneurysm occurs when a small hole in the vessel wall allows blood to leave the vessel and form a clot (hematoma) in the adjacent tissue space. In color flow, a pseudoaneurysm can be identified by its morphology and the presence of pulsating flow in the hematoma (within the part of the hematoma that has not yet clotted). Pulsation in color flow is apparent by the rhythmic change in colors corresponding to the pulse rate.

Venous valves can become damaged leading to serious circulation problems. The main difference of the hemodynamics of the venous system compared to the arterial system is that the venous system is a lower-pressure, slower-velocity, and more constant-flow state. There are two features of the venous circulation that help move blood from the lower extremities (e.g., legs) to the heart which are needed to overcome gravitational forces, especially when standing. The first feature is the routing of many veins through leg muscles; when the muscles contract, they squeeze blood and apply pressure, forcing blood to flow. The second feature is unidirectional valves within veins, which only allow flow toward the heart. If a valve is malfunctioning due to disease or injury, it is termed incompetent, meaning it does not completely seal on application of back pressure. This permits venous blood to flow away from the heart, resulting in pooling of blood in the lower legs and feet, a condition known as venous insufficiency. Valvular incompetence can be seen on the Doppler spectrum by reflux flow (transient flow in the wrong direction) through the valve. Manual compression or a Valsalva maneuver performed by the patient may be used to help make the reflux more apparent.

Abnormal Vessel Development

An arteriovenous (AV) fistula is an abnormal direct connection between an artery and a vein, without the usual routing through a capillary. An AV fistula may be congenital or caused by trauma, sometimes as a complication in a procedure such as cardiac catheterization. Smaller AV fistulas, especially those further away from the heart, are often asymptomatic and do not cause problems. Larger AV fistulas can interfere with circulation to an organ or system and lead to serious complications such as blood clots and heart failure. The fistula provides a low-resistance pathway from the artery to the vein; therefore, the Doppler spectrum of the artery proximal to the fistula often exhibits a low-resistance pattern with spectral broadening. Near the fistula, a high pressure gradient between the artery and the vein causes high velocities. In the vein, the Doppler spectrum often shows turbulent flow at the fistula outlet and pulsatile features distal to the fistula.

The collateral circulation refers to smaller vessels that serve as alternate conduits of flow for vessels around and through various tissues. In general, in their native state, they are very small and pass marginal to no flow. When an occlusion occurs in a vessel they surround, a change occurs in the corresponding collaterals. The process

of how this change occurs is still under study (Chilian et al. 2012). However, in general the collateral widens as its internal pressure increases, and blood starts to move through it to bypass the obstruction. Detection of blood flow in a smaller vessel (by color flow imaging) near a vessel with abnormal flow patterns can serve as a secondary verification of an occlusion.

Emboli or Thrombi in Blood

A thrombus is a solid mass formed as a result of the clotting mechanism and is made up of platelets, fibrin, and other blood components. Clotting is a natural response to injury and may happen at the site of damaged endothelial cells, which line the inner wall of the blood vessel. In addition, thrombi tend to form at regions of slow-moving or sluggish blood flow, such as are found at eddy sites. As a thrombus grows, it can partially obstruct a vessel leading to similar high-resistance flow patterns in the Doppler spectrum as described above for stenosed blood vessels. In ultrasound imaging, newer thrombi are less echogenic and thus harder to see; they may be detected by the inability to completely compress the vessel (if a vein) and the partial or complete absence of flow in the vessel. In addition, the thrombus may partially break away from the vessel wall and move downstream, where it is likely to encounter a vessel with a smaller diameter and obstruct it. An example of a particularly dangerous condition is deep vein thrombosis (DVT), which occurs when a thrombus forms due to low mass flow rate of blood because of incompetent venous valves, long periods of sitting, or a family history of clotting. If this thrombus breaks away from its attachment site, it can move through the right side of the heart to the pulmonary circulation, eventually blocking a vessel leading to the lungs, where it is known as a pulmonary embolism, a very serious condition.

An embolus is an abnormal matter (usually gaseous or particulate) freely circulating in the blood. Often they consist of pieces of thrombi that have broken away from the attachment site, or bits of plaque that have broken away from an atheromatous site (a site where abnormal fatty deposits have developed). They may also be inadvertently introduced into the systemic circulation during surgery and consist of tissue fragments, air bubbles, platelet thrombi, fibrin plugs, or microscopic flakes of catheters and vascular tubing. Emboli have a singular Doppler signature, consisting of very brief, broad-spectrum, high-energy “chirps” that overlay on the normal blood flow waveform. In situations where they have a known chance of occurring (such as monitoring), they are often referred to as high-intensity transient signals (HITS).

Potential Applications to Prognosis, Other Diseases, or Conditions

A number of ultrasound blood flow and tissue motion detection strategies have been investigated in research labs for decades. This section describes two such areas which have been gradually making their way into the clinical realm and show much promise for widespread acceptance in the future: multidimensional blood flow detection and elasticity imaging.

As described before, most commercial machines today utilize, and most clinical practitioners are trained on, one-dimensional blood flow analysis, i.e., one-dimensional vector velocity. At a given time and location in space, both the direction (one-dimensional – toward or away from the transducer) and the speed of the blood velocity may be estimated. A more technical way of saying this is that only the 1D projection of the blood velocity vector along the axial direction of the ultrasound beam is estimated. The velocity field is rarely constant across a transverse section of a blood vessel, especially in an artery or anywhere where pathology is present. Therefore, a two- or three-dimensional blood flow estimator would give more information to the physician, as it is the mass transport of oxygen which is usually of more importance than the blood velocity. Here, just a few of the many ideas generated throughout the years are briefly outlined.

The idea of using multiple (two or three) transducers to independently acquire estimates of velocity vectors in two or three spatial dimensions reaches back at least to 1978 (Fox 1978). This idea has evolved over the years to the general concept of using beamforming to make velocity estimates along beams steered at different angles, forming a 3D velocity estimate through a vector sum (Phillips et al. 1995; Dunmire et al. 2000; Tortoli et al. 2010). This method has been shown to accurately measure peak systolic velocities in both healthy and stenosed carotid arteries (Lenge et al. 2014).

Another multidimensional method is two- or three-dimensional tracking of the speckle pattern. This method measures blood or tissue motion by cross-correlating spatial echo patterns ("speckle") formed by coherent interference appearing if many scatterers exist within an area or volume smaller than the resolution limit (which is always the case within a blood vessel). This method was applied to blood flow in one dimension as an alternative to Doppler in the 1980s (Embree et al. 1985) and shortly thereafter to two dimensions (Trahey et al. 1987). This method did not suffer from aliasing but was computationally intensive; many variations were introduced over the years to help reduce the processing power required. For example, feature tracking is an alternative to speckle tracking which tracks only features of the speckle pattern, greatly reducing the complexity and making 3D blood flow estimation possible (Bashford and von Ramm 1996; Xu and Bashford 2009; Kuo and von Ramm 2008). However, a more important development has been recent optimization (though using earlier ideas) of technical beamforming improvements, in particular the combination of plane wave imaging on transmit (Sandrin et al. 1999) and parallel beamforming on receive (Shattuck et al. 1984) (for a review, see (Cikes et al. 2014)). These advances have enabled 2D speckle tracking and its variants to be used in real time to visualize complex heart flow patterns (e.g., Angelelli et al. 2014; Takahashi et al. 2014).

A third technique is to use a novel method in the lateral direction, i.e., perpendicular to the ultrasound beam, coupled with a conventional Doppler (or closely related) technique in the axial direction. The reasoning is that the Doppler technique has generally worked well for axial flow (parallel to the ultrasound beam axis), while flow in the lateral direction is theoretically not detectable using Doppler. One such method involves modulating the field spatially in the lateral direction using

transducer beamforming techniques (Anderson 1998; Jensen and Munk 1998). As blood moves laterally, it passes through a spatially varying beam which reveals its lateral motion upon reception. This method has been used recently to estimate volume flow (by multiplying 2D velocity estimation by vessel cross-sectional area) in AV fistulas (Hansen et al. 2014). A different lateral-flow method involves comparing the lateral spread (or "stretch") of the speckle pattern created when moving scatterers travel through the acoustic field (Xu and Bashford 2010, 2013). This method takes advantage of the very small time delay between A-lines which are formed during creation of the B-mode image. The relationship between the lateral velocity of the scatterers and the scan speed governs the resulting spatial correlation of the speckle pattern. This method has been used recently to measure lateral flow in the common carotid artery (Xu et al. 2014).

Finally, the flourishing field of elasticity imaging, though not strictly blood flow detection, is so closely related it deserves to be briefly mentioned here. Very generally speaking, elasticity imaging encompasses a range of techniques that observe the motion of tissues (strain) due to the application of a mechanical force (stress). The relationship between the two can be used to estimate the elastic modulus, i.e., the tissue stiffness. Tissue stiffness has been shown to be an important biomarker of various disease pathologies; the ability to quantitatively assess stiffness noninvasively is a significant advance in medicine. The close relationship to blood flow detection is in two areas: first, monitoring the tissue strain (motion) uses many of the same techniques described in this chapter to assess tissue motion. Second, the entire cardiovascular system is highly dependent on the elasticity of vessel walls and cardiac muscle. Elasticity imaging has the potential to become a common adjunct to conventional echocardiography. The interested reader is encouraged to consult recent reviews, e.g., Barr (2014) and Sarvazyan et al. (2013).

Summary Points

- The cardiovascular system is one of the most crucial systems in the body due to the rapid development of life-threatening situations that may occur in pathologic conditions.
- Ultrasound is an ideal mechanism for sensing blood flow due to its safety, noninvasive and painless function, real-time operation, flexibility in spatial/temporal resolution control, and relatively inexpensive cost.
- There are three main modes of ultrasound blood flow detection in wide use today: color flow Doppler, PW Doppler, and CW Doppler. Of these three, only CW Doppler uses the true physical Doppler effect.
- Color flow Doppler measures a single parameter (mean blood flow velocity) over a two-dimensional region of interest. PW Doppler is complementary to color flow Doppler, assessing the velocity distribution (essentially a histogram of velocities) at a single resolution-limited point in space.

- All three of the main modes of blood flow detection measure a one-dimensional projection of the blood flow velocity vector along the ultrasound beam axis rather than true volume blood flow.
- Many factors affect the Doppler spectra; major parameters clinicians look for include pulsatility, resistance, peak velocities, and energy.
- Doppler spectral patterns can reveal normal and pathological conditions both at the site of interrogation, and/or at proximal and distal sites in the circulation which have secondary effects at the site of interrogation.
- Two techniques that use flow (motion) estimation as a central tool and show promise for widespread acceptance into mainstream use are multidimensional blood flow estimation and elasticity imaging.

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Myocardial Blood Flow as a Biomarker

45

Uttam Shrestha and Youngho Seo

Contents

Abstract

Myocardial blood flow (MBF) is an emerging and important biomarker of cardiovascular disease. Its absolute quantification in physical unit (ml/min/g) is challenging and has been a high-priority translational research topic for assessing coronary artery disease (CAD) for decades. Currently, dynamic positron emission tomography (PET) is considered as a standard noninvasive imaging technique to measure MBF. Other noninvasive imaging modalities including single photon emission computed tomography (SPECT), X-ray computed tomography (CT),

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_25

and magnetic resonance imaging (MRI) have also been utilized for measuring MBF. The present chapter reviews the measurement of MBF using these techniques as well as its significance in a number of clinical applications.

Keywords

Myocardial blood flow • Myocardial perfusion • PET • SPECT • CT • MRI • Myocardial perfusion reserve • Coronary flow reserve • Coronary artery disease • Myocardial infarction • Myocardial hibernation • Cardiomyopathy • Coronary steal

Abbreviations

ATP	Adenosine triphosphate
BPI	Brain perfusion imaging
CA	Coronary angiography
CAD	Coronary artery disease
CBF	Cerebral blood flow
CFR	Coronary flow reserve
CMR	Cardiovascular magnetic resonance
CRT	Cardiac resynchronization therapy
CS	Coronary steal
CT	(X-ray) computed tomography
CTCA	Computed tomography coronary angiography
DCE	Dynamic contrast enhanced
DCM	Dilated cardiomyopathy
DE	Delayed enhancement
ECG	Electrocardiogram
EPS	Electrophysiological study
FDA	Food and Drug Administration
Gd	Gadolinium
HCM	Hypertrophic cardiomyopathy
LAD	Left anterior descending
LCX	Left circumflex artery
LVH	Left ventricular hypertrophy
MBF	Myocardial blood flow
MH	Myocardial hibernation
MP-CT	Myocardial perfusion computed tomography
MPI	Myocardial perfusion imaging
MPR	Myocardial perfusion reserve
MR	Magnetic resonance
MRI	Magnetic resonance imaging
PET	Positron emission tomography
QCT	Quantitative computed tomography
RCA	Right coronary artery
SPECT	Single photon emission computed tomography

TAC	Time-activity curve
TIC	Time-intensity curve

Key Facts

- Coronary artery disease (CAD) can be assessed using noninvasive imaging techniques.
- When there is an obstruction in the coronary blood vessels, the blood flow is typically impaired.
- By noninvasive imaging techniques, the blood flow in the tissue of heart muscle (myocardial blood flow or MBF) can be quantitatively measured.
- The impairment of myocardial blood flow during resting or stress conditions can be revealed by quantitative measurement of MBF in its physical unit (ml/g/min).
- In addition to qualitative assessment of myocardial perfusion, absolute MBF measurement should add important information for the management of a number of coronary artery diseases.

Definitions

Compartment model Mathematical description of how a tracer behaves in the body by assigning many parts of the body into a limited set of compartments depending on the function such as input compartment (e.g., tracer activity in the arterial blood) and target compartment (e.g., tracer activity in myocardium).

Coronary artery disease Typically a disease that contributes to narrowing of blood vessels (stenosis) that provide oxygenated blood to the heart (i.e., coronary arteries).

Coronary flow reserve (CFR) CFR is the maximum amount of blood that can pass through myocardium during a stress condition when excessive oxygenated blood is required with respect to the baseline amount of blood during a resting condition. It is generally measured as the ratio of MBF during stress to that at rest. The normal minimum CFR value in healthy human is approximately two.

Dynamic perfusion imaging Using positron emission tomography (PET), single photon emission computed tomography (SPECT), X-ray computed tomography (CT), or magnetic resonance imaging (MRI), dynamics of tracers (radioactive perfusion imaging agents for PET and SPECT or contrast agents for CT and MRI) is measured over time by taking the images in multiple time frames.

Myocardial blood flow (MBF) MBF is the amount of blood perfused per unit time per unit mass of the tissue of the heart muscle (myocardium). It is different from

blood flow in coronary arteries, which is the volume of blood flow in the coronary blood vessels per unit time.

Introduction

The human heart pumps the blood throughout the body through physiological process, called circulation. In order for the heart to pump the blood, it requires strong muscle known as myocardium. The heart relies almost exclusively on the aerobic oxidation of substrates to generate the required energy. Thus the viability and function of the heart depend upon a delicate balance of oxygen supply and demand. The myocardium maximally extracts oxygen delivered from arterial blood at rest. With exercise, the tissue autoregulates the resistance of vascular bed and obtains essential supply of oxygen by increasing coronary blood flow.

A clear understanding of the coronary artery anatomy and distribution of blood flow to the myocardium is necessary to understand pathophysiology of the heart. Oxygen to the myocardium is delivered through three main coronary arteries: the left anterior descending (LAD) coronary artery, right coronary artery (RCA), and left circumflex artery (LCX). Oxygenated blood through these arteries passes down to arterioles and capillaries to cellular mitochondria where oxidative phosphorylation occurs that results in the production of adenosine triphosphate (ATP), a main currency of cellular energy. When coronary blood flow does not increase to a level sufficient to meet its demand, there is a mismatch between oxygen demand and supply, aerobic metabolism is impaired, and a clinical condition known as ischemia develops. Numerous factors such as metabolic, anatomic, hormonal, endothelial, and coronary perfusion pressure determine the control of myocardial blood flow (MBF) in the heart.

Although blood flow in the coronary artery and myocardial blood flow are proportional, they represent, in principal, two different quantities. The tubular blood flow in the coronary arteries is the amount of blood that passes through per unit time and measures approximately 250 ml/min during the resting stage in normal subjects (Guyton and Hall 1996). When arteries are in contact with the tissue such as the myocardium, they branch to tiny blood vessels like arterioles and capillaries that supply blood to cells via perfusion. The perfusion of the blood in myocardium, which is the so-called myocardial blood flow, is much less than the tubular flow in coronary arteries depending upon the mass of the tissue and thus measured in flow (ml/min) per gram of tissue. Because of this reason, the absolute measurement of the MBF in its physical unit (ml/g/min) is technically challenging.

It is noteworthy that the main coronary arteries normally contribute <5 % of the coronary vascular resistance. A noticeable arterial blood flow is reduced with the diameter of the vessel only if the lesion is significant. An invasive method such as coronary angiography (CA) suggests that a stenosis lesion <50 % diameter corresponding to a 75 % luminal cross section is clinically insignificant (see

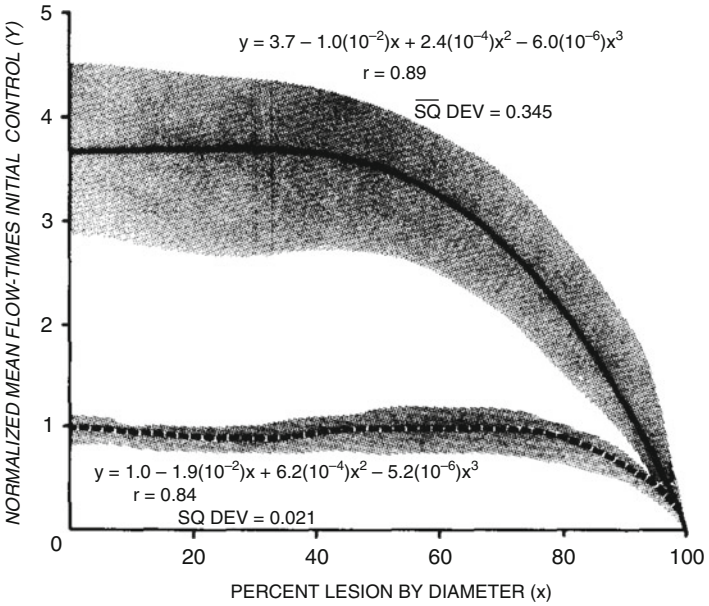


Fig. 1 Normalized MBF as a function of percent coronary stenosis in resting state (*dotted line*) and during hyperemia after intracoronary injection of Hypaque (*solid line*) in the canine model. The normal MBF do not show any significant change in resting flow for stenoses <80 %, but the reduction of hyperemic flow becomes apparent in the presence of coronary stenoses >50 % (Reprinted with permission from Gould et al. 1974)

Fig. 1). However, symptoms of angina at rest may appear when the lesion with a diameter >80 % or a luminal cross section of 96 % appears (Gould et al. 1974).

Myocardial blood flow is also associated with other disease states. Diabetic patients have been shown to have abnormal blood flow due to distortion of microvasculature capillary basement. Similarly, cardiomyopathy with left ventricular hypertrophy (LVH) or idiopathic dilated cardiomyopathy also impairs function of the heart and cannot pump blood efficiently resulting in a blunted regional MBF.

Myocardial perfusion imaging (MPI) is a noninvasive and qualitative method to assess the MBF in the tissue. In addition to a standard noninvasive method of MPI based on single photon emission computed tomography (SPECT), basically, all noninvasive perfusion imaging techniques including magnetic resonance imaging (MRI), positron emission tomography (PET), and X-ray computed tomography (CT) can be used for evaluating myocardial perfusion.

In contrast to MPI using SPECT, the standard noninvasive method of absolute MBF measurement is based on PET. Nevertheless, there have been active developments using all other three-dimensional imaging modalities primarily including SPECT, MRI, and CT for calculating absolute MBF. As more methods of noninvasive evaluation of MBF are becoming available, there also have been a number of

potential clinical applications proposed, related to the correlation of epicardial and microvascular coronary pathophysiology with MBF measurements.

Arterial Blood Flow Versus Myocardial Blood Flow

MBF or more precisely regional MBF measured in unit of ml/min per gram of tissue is now becoming one of the clinical indicators of cardiovascular disease. Noninvasively measured perfusion-based flow estimates suggest that MBF in healthy human can vary widely from as low as 0.5 ml/min/g at rest to 5 ml/min/g during stress (Gewirtz et al. 2002; Chareonthaitawee et al. 2001); its hyperemic value in animal may be as large as 8.5 ml/min/g for exercising swine (Clair et al. 1998) and 9.3 ml/min/g for dipyridamole-administered ponies (Parks and Manohar 1983). During stress, boosting MBF via either coronary vasodilation and/or decrease of coronary vascular resistance is a principal compensatory mechanism for increased myocardial oxygen demands (Bassingthwaighte et al. 2001). When there is any disease in the coronary arteries, and further down in microvasculature, the blood flow in myocardium could be affected. MBF could therefore be considered an important biomarker for heart disease.

MBF Measured by Dynamic PET

Presently, dynamic PET is a clinical workhorse for the measurement of absolute myocardial blood flow noninvasively (Bergmann et al. 1989; Shah et al. 1985; Manabe et al. 2009; Lortie et al. 2007; Lee et al. 2005; Ahn et al. 2001; Wu et al. 1995; Choi et al. 1993). Several techniques employing positron-emitting radionuclides such as $^{13}NH_3$ (^{13}N-ammonia) (Shah et al. 1985; Choi et al. 1993), $H_2{}^{15}O$ (^{15}O-water) (Bergmann et al. 1989; Ahn et al. 2001), and ^{82}Rb (Manabe et al. 2009; Lortie et al. 2007) with appropriate tracer kinetic modeling have been proposed. ^{15}O-water is an inert and freely diffusible tracer with a linear extraction with the flow and the most desirable for the quantification of MBF. However, a poor tissue to blood contrast ratio and limited count rate make this tracer less usable for the clinical application of the detection of coronary artery disease (CAD). On the other hand, PET with ^{13}N-ammonia has been validated with microsphere for the measurement of MBF and widely used because it provides a superior image quality with clear delineation of blood-endocardium wall. Although the use of ^{13}N-ammonia dynamic PET for the quantification of MBF has been compromised by the nonlinear tracer extraction for higher blood flow, it is considered a means of detecting CAD and other pathophysiological conditions of the heart noninvasively.

The standard technique of measuring MBF using dynamic PET consists of calculating regional perfusion from the tomographic data by fitting the tissue time-activity curve (TAC) to a compartment model based on the kinetics of the specific tracer given that the atrial input function is a priori information. In order to enhance the accuracy of the method, both blood pool (plasma) and myocardial activity curves

are to be corrected for partial volume effect and contamination from spillover. A prior condition of the application of this method is to obtain the exact atrial input function from the sequence of dynamic images. In order to calculate absolute MBF, a nonlinear flow-extraction correction (Salerno and Beller 2009) has to be introduced, and the tracer uptake should be separated from the metabolite for radionuclide such as ^{13}N-ammonia (Hutchins et al. 1990).

MBF Measured by Dynamic SPECT

Despite the fact that dynamic PET is a well-established tool for the quantification of MBF both in humans and animal models, its routine clinical use is still questionable due to many factors including short physical half-lives of positron-emitting radionuclide, requirement of an on-site cyclotron (for ^{13}N-ammonia), high cost of maintaining Sr-82/Rb-82 generator, and limited accessibility of PET scanners (Schelbert 1992, 2014). SPECT MPI has been a widely used diagnostic technique for decades for the detection of ischemia and perfusion-related coronaropathy (Mariani et al. 2008; Rahmim and Zaidi 2008). The most commonly used radionuclides in SPECT MPI are thallium 201 (Tl-201), ^{99m}Tc-sestamibi, and ^{99m}Tc-tetrofosmin with typical first-pass extraction of 86 %, 64 %, and 54 % in humans, respectively, at resting myocardial blood flow levels (i.e., ∼1 ml/g/min). The extraction is considerably lower at high-flow values such as in exercise-induced coronary vasodilation with the MBF that is two- to fivefold from the resting baseline. In addition to diffusion-limited roll-off of the radionuclides, the low sensitivity, low collimator response, and low efficiency due to limited spatial and temporal resolution of the SPECT system (Gullberg et al. 2010) had made it impracticable for the use of quantifying MBF. However, significant progresses have been already achieved in recent years with new dynamic acquisition protocols (Zan et al. 2013; Reutter et al. 2002). Recently, with the advent of new dedicated cardiac cameras such as D-SPECT from Spectrum Dynamics or the Discovery NM 530c/570c cameras from GE Healthcare, there is a renewed interest among scientific community to measure MBF using SPECT using existing radiotracers such as ^{99m}Tc-sestamibi, ^{99m}Tc-tetrofosmin, and ^{99m}Tc-teboroxime (Garcia 2014; Slomka et al. 2014; Klein et al. 2014; Wells et al. 2014; Koshino et al. 2014). An improved myocardial extraction and relatively long retention was obtained with a new class of perfusion imaging agent known as ^{123}I-iodorotenone (Broisat et al. 2011) over a wide range of flow values, and its isomers were further evaluated recently in animal models (Wei et al. 2014).

Unlike PET, the measurement of MBF with SPECT is still in its infancy with limited number of publications. Recent studies with a dedicated cardiac camera (D-SPECT, Spectrum Dynamics) have shown that it is possible to obtain the myocardial perfusion reserve (MPR) (Ben-Haim et al. 2013), an analogue of coronary flow reserve (CFR), for the assessment of patient prognosis. Another study in porcine model tested the clinical feasibility of the usability of commonly available SPECT radionuclides (Tl-201, ^{99m}Tc-sestamibi and ^{99m}Tc-tetrofosmin) for the

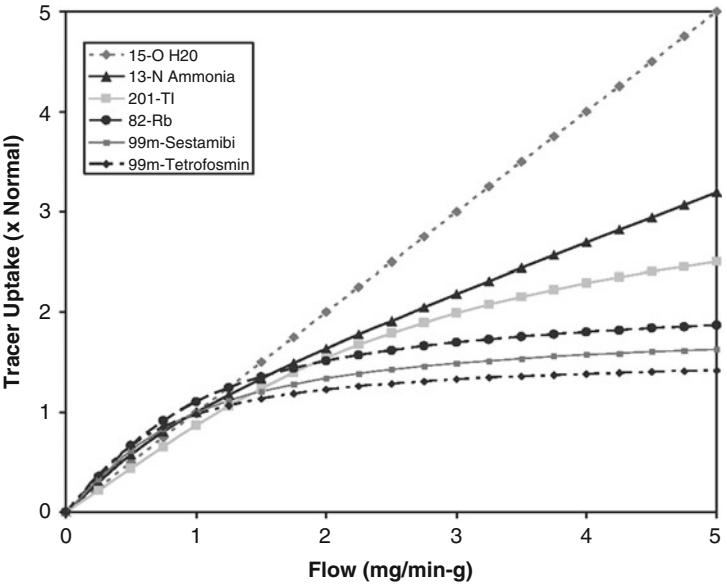


Fig. 2 Myocardial tracer uptake as a function of myocardial blood flow for commonly used both SPECT and PET perfusion agents. There is a significant roll-off phenomenon at high-flow rates for ^{99m}Tc-sestamibi, ^{99m}Tc-tetrofosmin and ^{82}Rb (Reprinted with permission from Salerno and Beller 2009)

measurement of absolute MBF using a multipinhole cardiac SPECT camera (Wells et al. 2014). There are also advances on dynamic iterative image reconstruction method with CT attenuation and scatter correction on a widely used dual-headed SPECT/CT scanner by modifying the traditional acquisition protocol. A major technical challenge of using tracers such as ^{99m}Tc-sestamibi and ^{99m}Tc-tetrofosmin is their extraction insensitivity for high myocardial blood flow values: the uptake almost flattens for high MBF values (see Fig. 2). This would suggest to use Tc-99m teboroxime, an agent approved by the US Food and Drug Administration (FDA) for clinical use. Tc-99m teboroxime exhibits very high myocardial extraction fraction. However, Tc-99m teboroxime has rapid myocardial washout and cumulative hepatic uptake, and conventional acquisition protocol may not be suitable for the measurement of MBF.

MBF Measured by Dynamic Perfusion CT

MBF is vastly influenced by the architecture of the intermediate blood vessels, known as arterioles. The tissue vascular bed containing arterioles are the site of metabolic regulation of MBF and responsible for the impairment of myocardial

perfusion and related abnormalities. The invasive coronary angiography (CA) and noninvasive coronary computed tomography coronary angiography (CTCA) are well-established and the current state-of-the-art imaging modalities for analyzing anatomic detail of the epicardial arteries in the detection of coronary stenosis and atherosclerosis but unable to resolve functional details of microvascular dysfunction and their physiological significance. Generally, the stenotic lesions (luminal cross section <50 %) are not flow limiting and are considered to be physiologically insignificant. However, the impact of such non-flow-limiting coronary stenoses in heart disease requires additional functional information. An emerging alternative of simultaneous visualization of coronary microanatomy and functional information is the perfusion-based contrast-enhanced CT scans. In fact, stress myocardial perfusion CT (MP-CT) has shown promises for providing combined anatomical and physiological information in diagnosis of CAD and left ventricular dysfunction.

In a nutshell, MP-CT protocol, in analogy to myocardial perfusion imaging (MPI) using SPECT or PET, consists of rest and pharmacologically induced stress acquisitions using stress agents such as adenosine, dipyridamole, or regadenoson with administered iodinated contrast agent. Areas of low-contrast attenuation proportional to tissue concentration signify poor perfusion due to ischemia or infarct and can be immediately mapped with anatomical detail to find the coronary stenotic lesion.

Perfusion CT has served as a fast diagnostic and noninvasive imaging modality in the functional-anatomical cardiology. Dynamic acquisition of perfusion CT could be also performed to derive time-attenuation curves by repetitive fast scans before and during the first pass of CT contrast agent. Assuming that the flow of the CT contrast agent directly represents the blood flow, applying the same one-tissue compartment principle of calculating the myocardial blood flow as using PET and SPECT is still applicable with time-attenuation curves in myocardium and an arterial compartment such as the left ventricular chamber. The absolute MBF measurements at both rest and stress conditions using dynamic perfusion CT also provide data for the CFR measurement. The dynamic perfusion CT imaging can be compared with perfusion cardiac MRI for the sensitivity and specificity measurement to detect perfusion defects. In the proof-of-concept study (Rossi et al. 2014), with CT coronary angiography and dynamic perfusion CT imaging in a cohort of 80 patients, it was concluded that the measured MBF index performed better than visual CTCA and quantitative CT (QCT) in the identification of functionally significant coronary lesions. Figure 3 demonstrates myocardial perfusion defects in the anterior and anteroseptal walls related to the LAD, as well as the inferior and inferoseptal wall related to the LCX.

MBF Measured by Dynamic Perfusion MRI

Magnetic resonance imaging (MRI) using an MRI contrast agent is also an imaging modality that provides a means of measuring absolute MBF as well. Like dynamic perfusion CT, cardiac MR perfusion imaging using a contrast agent could be

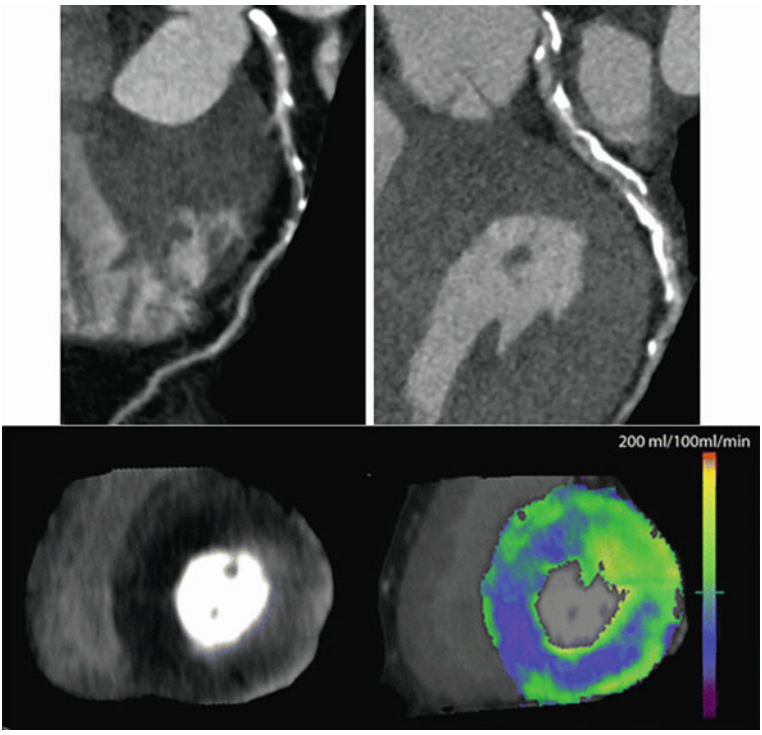


Fig. 3 Example images of the heart in a 60-year-old man with typical angina showing extensive highly calcified plaques in the LAD (*top left*) and in the LCX (*top right*). CT perfusion image (*bottom*) shows a perfusion defect in the anterior, anteroseptal, inferoseptal, and inferior myocardial wall (Reprinted with permission from Rossi et al. 2014)

obtained dynamically. This dynamic contrast-enhanced (DCE) MRI is a method that has shown its utility mostly in cancer imaging (e.g., breast and prostate). In cardiac MRI, the delayed contrast enhancement (DCE, the same acronym as for dynamic contrast enhanced, or simply DE) provides the information on the myocardial perfusion, and when perfusion MRI before and during the first pass is acquired dynamically, the dynamic data provides time-intensive curves that in turn could be plugged into the same one-tissue-compartment model to derive absolute myocardial blood flow. The mathematical modeling using compartments for cardiac dynamic perfusion MRI is also based on an assumption that the MR contrast agent during the first pass behaves like a freely diffusible tracer such as water. In addition, the somewhat sophisticated mathematical modeling for the one-tissue-compartment model could be replaced by a simplified the mathematical model to easily derive MBF as explained below.

Gadolinium (Gd)-based MRI contrast agent is infused intravenously. The cardiac perfusion MR images are acquired repeatedly at every cardiac cycle before and

during the first pass of Gd-based MRI contrast agent. The decreased T1 relaxation time from the Gd contrast will be visualized in Gd-rich (i.e., perfusion-rich) myocardial tissues as T1-weighted signal intensity increases. In the area of low blood flow (i.e., less Gd contrast), the T1-weighted signal intensity is reduced. Cine cardiac MRI is performed in addition to delayed contrast enhancement. The time portion of the cine (i.e., dynamic) cardiac MRI during the first pass of the contrast infusion is used for deriving myocardial blood flow (Lee and Johnson 2009).

A simplified technique of MBF calculation based on cardiac MRI exists using two bolus injections assuming the circulation is a shift-invariant system (Christian et al. 2008). The assumption that the circulation systems remain the same between these two time points provides theoretical framework for the low and high concentrations of contrast agent (but keeping the same volume) injected at two different time points. The low-concentration bolus injection is used to derive arterial input function based on time-intensity curve (TIC) from the arterial compartment such as the left ventricular chamber, and the high concentration of contrast agent is used to derive intensive enhancement over time from the myocardial regions. With the two TICs, of course, the same one-tissue-compartment model could be applied; however, for simplification, the differential equation from the one-tissue-compartment model could be rewritten as a convolution function. Mathematically, the signal enhancement in the myocardium over time (i.e., intensity function of time) is a convolution of an arterial input function and a shaped function that is generalized as a Fermi function (Christian et al. 2008). With the acquired arterial input function and the intensity function in myocardium, when the deconvolution is applied, the Fermi function could be derived as a one-step process. The peak amplitude of the fitted Fermi function becomes absolute myocardial blood flow (see Fig. 4).

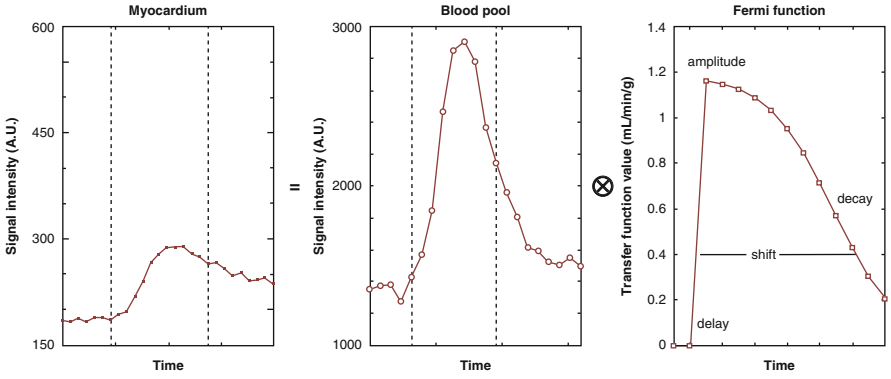


Fig. 4 The time-intensity curve (*TIC*) in the myocardial tissue equals to the TIC in the blood pool convoluted with a Fermi transfer function with a certain shape. The absolute myocardial blood flow (*MBF*) is the peak amplitude of the fitted Fermi function. The area between the *dashed lines* of the blood pool TIC plot (*middle*) is used for fitting the Fermi transfer function. *AU* arbitrary unit (Reprinted with permission from Lee and Johnson 2009)

Major Applications of MBF in the Disease Control

Multivessel Coronary Artery Disease and MBF

The vasomotor mechanism is a myogenic response in myocardium that autoregulates the blood flow in change of perfusion pressure according to metabolic needs (Duncker et al. 2014). In normal condition, arteries with insignificant stenotic lesion (luminal cross section <75 %) do not offer apparent resistance; autoregulation mechanism with metabolic vasodilator relaxes small resistance vessels to maintain the distal coronary perfusion pressure and flow reserve intact. However, in the presence of critical stenotic lesion (luminal cross section >95 %), a substantial fraction of resistance resides near the proximity of the lesion, the post-stenotic pressure drops significantly, and the flow reserve becomes exhausted resulting myocardial ischemia. In multivessel CAD, although lesions can be pathological, the conventional MPI do not differentiate normal to abnormal tissue due to homogeneous distribution of tracer activity.

An example of detecting CAD by measuring the absolute values of MBF is shown in Fig. 5.

Hibernating Myocardium and MBF

Myocardial hibernation (MH) (Bonow 1995), a state of decreased contractile activity and metabolic demands in a segment of ischemic myocardium poorly perfused by a stenotic coronary artery and dysfunctional, yet viable, is a signature of sustained reduction of blood flow.

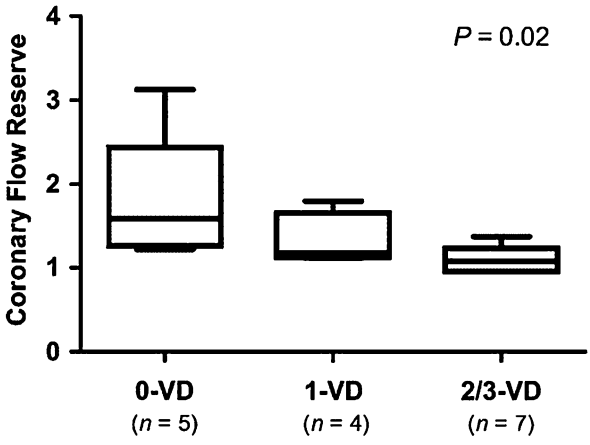


Fig. 5 Quantification of a retention index to describe myocardial perfusion reserve using a dedicated solid-state cardiac camera. Global MPR index was higher in patients with normal MPI ($n = 51$) than in patients with abnormal MPI. Shown is the Global MPR index by angiographic disease (*VD* vessel disease stenosis ≥50 %) (Reprinted with permission from Ben-Haim et al. 2013)

MH and myocardial stunning are the conditions that may coexist in patients with ischemic heart disease.

The impairment in perfusion reserve as well as resting flow has been recognized in hibernating myocardium (Hickman et al. 2010). It was suggested that a successive progression from stunning characterized by normal flow but reduced CFR to diminished resting flow is an indicator of MH. Therefore regional MBF can be a true biomarker for the identification of viable myocardium since a naïve comparison of MBF between remote to proximal would delineate semi-functional tissue that can be diagnosed with electrocardiogram (ECG) or MRI.

Coronary Steal Syndrome and MBF

Coronary steal (CS) syndrome refers to a generic heart condition in which an increase in blood flow in stress, due to vasodilation, to a certain area with already well-perfused myocardium causes a decrease in flow to another area of the myocardium that is supported primarily by collateral circulation (see Fig. 6). Myocardial ischemia due to CS is generally believed to be manifested clinically by measuring a

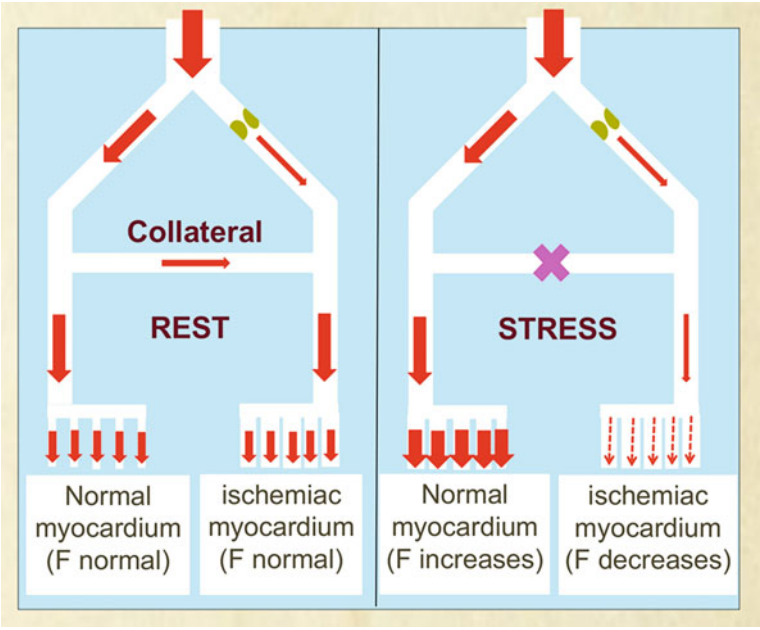
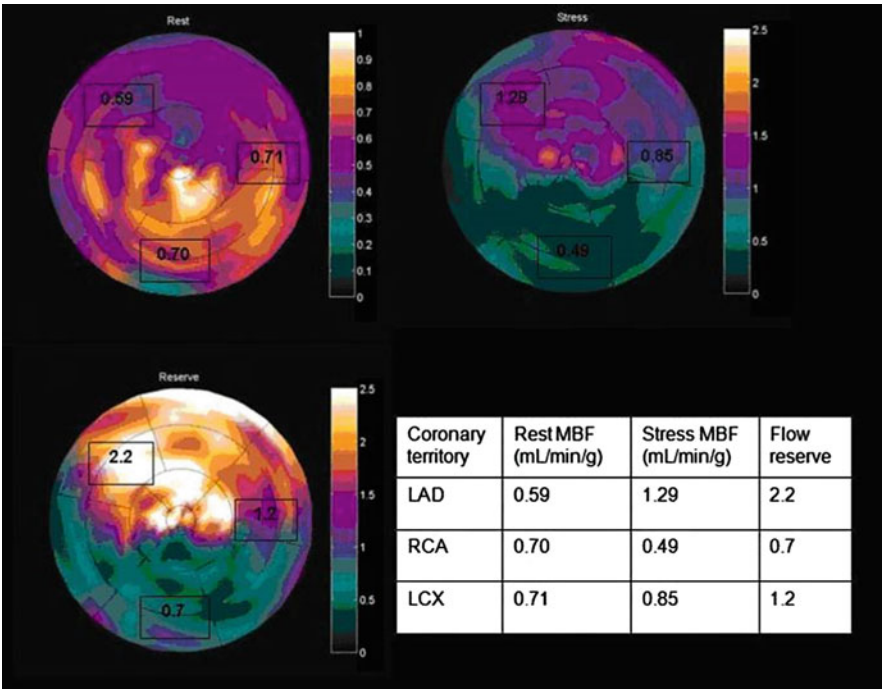


Fig. 6 Schematic diagram of coronary steal syndrome in collateral circulation at rest (*left*) and during stress (*right*)

pressure drop proximal to the collateral origin during pharmacologically induced hyperemic flow. In routine clinical practice, however, coronary steal syndrome cannot be not easily detected. The SPECT/CT, PET/CT, or perfusion CT with dynamic acquisition could enable both measurement of absolute MBF and visualization of coronary anatomy and thus identify the diagnosis of CS syndrome. Although CS has been common to many CAD patients, there are limited reports that suggest its absolute magnitude (Heijne et al. 2010) (Akinboboye et al. 2001).

Figure 7 depicts a typical polar map of absolute myocardial perfusion (mL/min/g) at rest (right upper quadrant), stress (left upper quadrant), and coronary flow reserve (left lower quadrant). The flow reserve in RCA is 0.7 that indicates the coronary steal as some of the blood from the inferior region supported by RCA flew to the regions supported by LAD and LCX.



Coronary territory	Rest MBF (mL/min/g)	Stress MBF (mL/min/g)	Flow reserve
LAD	0.59	1.29	2.2
RCA	0.70	0.49	0.7
LCX	0.71	0.85	1.2

Fig. 7 Polar map of absolute myocardial perfusion (mL/min/g) at rest (*right upper quadrant*), stress (*left upper quadrant*), and coronary flow reserve (*left lower quadrant*). A decreased flow reserve is displayed in the inferior, inferolateral, and inferoseptal wall. The flow reserve in RCA is 0.7 implying coronary steal syndrome (Reprinted with permission under the "Creative Commons Attribution Noncommercial License" from Heijne et al. 2010)

Cardiomyopathy and MBF

It has been long known that patients with dilated cardiomyopathy (DCM) and other cardiac abnormalities independent of hemodynamic factors and epicardial CAD exhibit impaired vasodilator responses to both metabolic and pharmacologic stimuli (Cannon et al. 1987; Nitenberg et al. 1985). These perfusion abnormalities were attributed to coronary microcirculatory dysfunction in vascular bed. Absolute regional MBF was measured using ^{13}N-ammonia PET during pacing-induced tachycardia and after dipyridamole infusion in patients with DCM, and observed that the MBF had lower mean value as compared with that from the controlled subjects (Neglia et al. 1995). Another study in patients with heart failure due to idiopathic DCM using velocity-encoded cine magnetic resonance imaging (MRI) reported that the MBF at baseline was not significantly different compared with healthy subjects, but CFR was impaired significantly (Watzinger et al. 2005). Similar method was implemented earlier to evaluate the MBF in patients with hypertrophic cardiomyopathy (HCM) (Kawada et al. 1999). All these results strongly suggest that the extent of MBF impairment in cardiomyopathic patients can be a diagnostic predictor and risk stratification of immediate cardiac events.

Figure 8 shows the late-time image of an 81-year-old male, a dilated cardiomyopathy patient acquired during dynamic SPECT for the measurement of MBF. The patient had history of chest pain with reduced ejection fraction. The measured MBF in all territories (LAD, RCA, and LCX) was <1 ml/min/g.

Cardiac Electrical System and MBF

Impaired hyperemic MBF is a signature of ill response of the heart to oxidative stress and is a major precursor of myocardial infarction and related death in ischemic

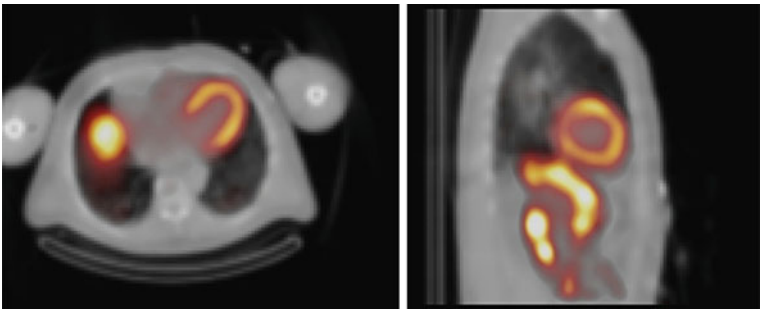


Fig. 8 Dynamic SPECT images of typical 81-year-old male patient with reported history of chest pain and reduced ejection fraction due to dilated cardiomyopathy

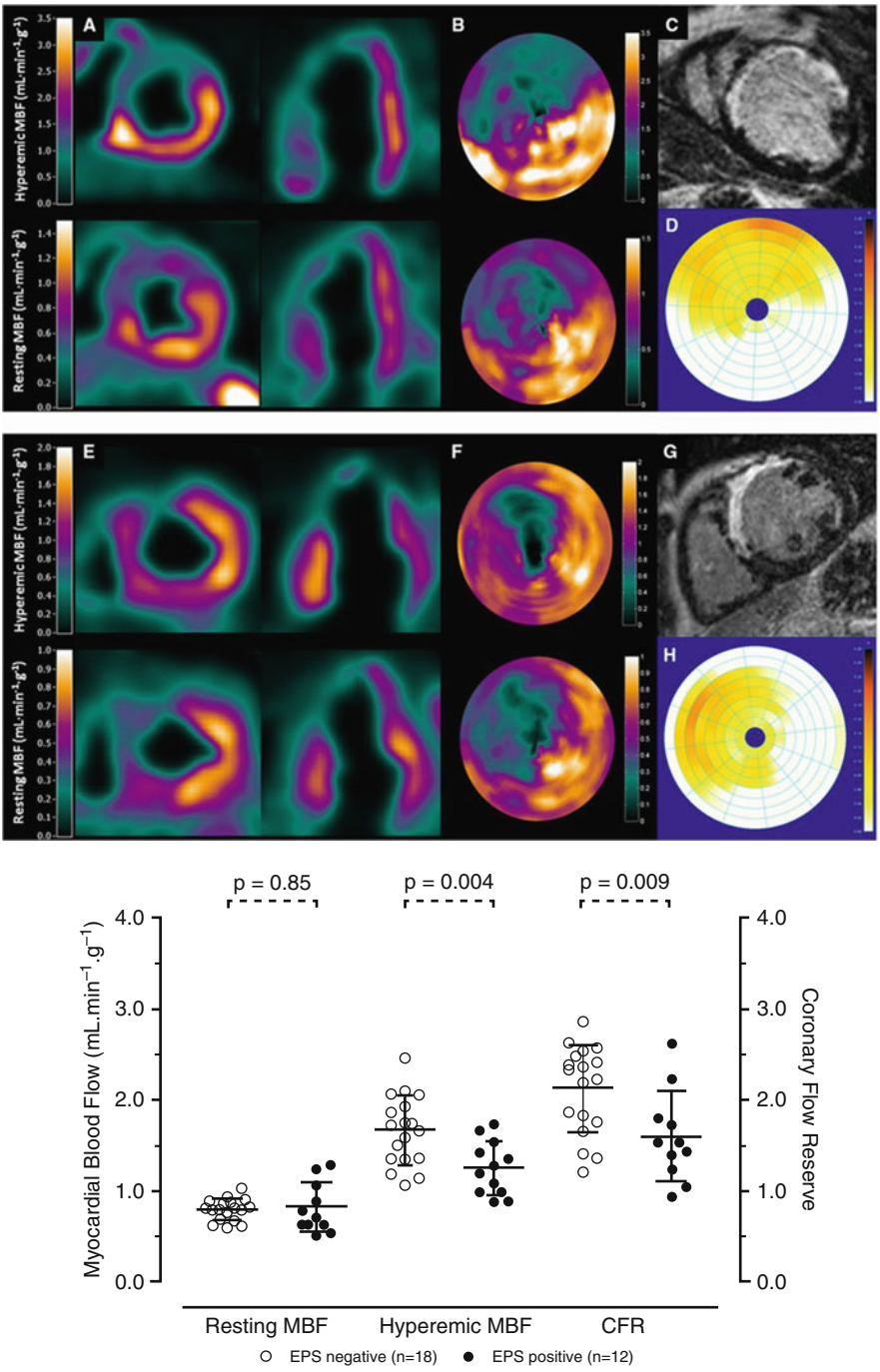


Fig. 9 (continued)

patients. But how the changes of MBF can be attributed to ventricular arrhythmia is still not clearly understood. Recent study conducted in patients with ischemic cardiomyopathy using ^{15}O-water PET showed reduced hyperemic MBF and CFR in positive electrophysiological study patients compared with negative electrophysiological patients (Rijnierse et al. 2014).

In a subgroup of patients with an asynchronous contraction pattern, it was demonstrated that cardiac resynchronization therapy (CRT) improved hemodynamic response of the heart with homogeneous utilization of oxygen consumption and thus improved MBF (Saxon et al. 2002). Although there were significant improvements in overall functioning of the heart after CRT implantation, a subgroup of patients (30–40 %) showed little to no response at all (Buck et al. 2009; van Hemel and Scheffer 2009). A huge controversy on how CRT affects myocardial efficiency and regional oxidative metabolism (Iyengar et al. 2007; Lindner et al. 2005; Ukkonen et al. 2003; Hamad et al. 2009; Nielsen et al. 2003) *made* this a new research topic in the management of heart disease, and as of now no data is available to relate the effect of CRT on measurement of absolute value of MBF (Fig. 9; Rijnierse et al. 2014).

Potential Applications to Prognosis and Other Diseases or Conditions

The measurement technique, especially mathematical foundations of myocardial blood flow, is generally applicable to other regional blood flow measurements such as cerebrovascular, renal, and hepatic system. Like cardiac MPI, perfusion is a downstream marker directly affected by blood flow, and there have been active efforts of cerebrovascular imaging focusing on brain perfusion. At present, the gold standard for measurement of regional cerebral blood flow (CBF) is based on dynamic PET brain perfusion imaging (BPI) using $H_2{}^{15}O$. Other imaging modalities, such as SPECT and MRI, are also being used, but all the modalities lack methods of calculating absolute values of CBF directly. Quantifying regional CBF is particularly challenging due to difficulty in measuring exact input function. Unlike myocardium, the vascular patterns of the kidney and liver are more intricate, and corresponding distribution of blood flow in these tissues is nonuniform, and the discussion is beyond the scope of this chapter.

Fig. 9 Positron emission tomography (*PET*) and cardiovascular magnetic resonance (*CMR*) images of patients with a history of an anterior wall myocardial infarction (*top*). Comparison of global resting myocardial blood flow (*MBF*), hyperemic MBF, and coronary flow reserve (*CFR*) between patients with positive and negative electrophysiological study (*EPS*) (*bottom*) (Reprinted with permission from Rijnierse et al. 2014)

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Cerebral Blood Flow Measurement for Neurological Assessments: Functional Transcranial Doppler Ultrasound

46

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_46

Abstract

Transcranial Doppler (TCD) is a special type of ultrasound developed to measure cerebral blood flow (CBF) and subsequently shown to be valuable in an increasingly larger number of clinical applications. Physically, TCD operates in the same way and is as safe as ultrasound imaging used during pregnancy. Changes in CBF in response to controlled stimuli have been shown to be valuable indicators of a variety of pathological conditions. In addition to measuring CBF, TCD can detect the passage of emboli in the bloodstream due to a stark difference in acoustic properties when emboli pass through the ultrasound beam. This unmistakable change in ultrasound signal echo between emboli and blood is due to density and acoustic impedance differences. The TCD signal is processed in real-time for visual display and measurement of cerebral blood flow velocities (CBFV). Two scanning techniques, the free-hand and continuous monitoring techniques, are the two primary methods for performing TCD. The free-hand technique is suitable for short-term monitoring (30 min or less) but is not effective for continuous monitoring due to operator fatigue and inconsistent transducer placement. The second method, continuous TCD monitoring, requires a fixation apparatus to hold the Doppler probe(s) in a static location at the scalp insonation site. Due to its low cost, real-time measurement capability, and easy portability, TCD has remained a valuable imaging tool when compared to more costly and stationary modalities. More recently, TCD has been used in conjunction with cerebral function tests (functional TCD, or fTCD) as a proxy for neuronal activity for indication in an ever-increasing number of brain studies. The future of TCD and fTCD as biomarkers for neurological assessment is exciting indeed.

Keywords

Transcranial Doppler • Cerebrovascular disease • Ultrasound • Emboli • Functional TCD • Autoregulation

Abbreviations

ACA	Anterior cerebral artery
AD	Alzheimer's disease
BCA	Basal cerebral artery
BH	Breath holding
CBF	Cerebral blood flow
CBFV	Cerebral blood flow velocities
CVR	Cerebrovascular reactivity
fTCD	Functional transcranial Doppler
ICA	Internal carotid artery
MCA	Middle cerebral artery
MCI	Mild cognitive impairment
MHz	Megahertz (one million cycles/s)
mW	milliWatts
mW/cm^2	milliWatts per centimeter squared (acoustic intensity)
PCA	Posterior cerebral artery

PI	Pulsatility index
RI	Resistivity index
SAH	Subarachnoid hemorrhage
TBI	Traumatic brain injury
TCD	Transcranial Doppler

Key Facts of Transcranial Doppler

- Despite the term "Doppler," TCD technology does not use the physical Doppler principle to detect blood flow.
- Transcranial Doppler can measure blood flow in the deepest parts of the brain; in fact, care must be taken when setting the depth to ensure flow information is coming from the correct side of the brain.
- TCD has no known side effects; it uses non-ionizing energy (mechanical sound waves) at very low power and is thus safe and painless.
- TCD can be used with fixation devices, allowing for measurement of blood flow even when subjects are moving.
- About 8 % of the population have temporal windows that attenuate sound too much to acquire a strong enough signal – research continues for overcoming this limitation.
- More complex forms of TCD machines can produce two-dimensional images of the flow in the brain, in addition to the velocity-time spectrum.

Definitions

Basal cerebral arteries The major arteries emanating from the base of the brain carrying and distributing the majority of blood flow to various lobes.

Circle of Willis A vascular "roundabout" fed by the internal carotid arteries and the basilar arteries and diverting blood to various areas of the brain via other basal arteries such as the MCA, PCA, and ACA.

Diastolic velocity (or end diastolic velocity) In one cardiac cycle, the blood flow velocity existing at the end of the relaxation phase of the heart, just before contraction. This value changes throughout the vasculature and is measured at a specific location by TCD.

Mean velocity In one cardiac cycle, the time-averaged velocity of the TCD envelope.

Pulsatility index (PI) The difference between peak systolic velocity and end diastolic velocity divided by the mean velocity.

Resistivity index (RI) The difference between peak systolic velocity and end diastolic velocity divided by the peak systolic velocity; a measure of the impedance the blood flow "sees" as it is flowing at the point of insonation.

Systolic velocity (or peak systolic velocity) In one cardiac cycle, the maximum blood flow velocity achieved due to the contraction phase of the heart. This value changes throughout the vasculature and is measured at a specific location by TCD.

TCD envelope The trace of maximum velocity at each point in time of a Doppler spectrum, from which hemodynamic parameters and indices are measured.

Introduction

First developed by Rune Aaslid in 1982 (Aaslid et al. 1982), transcranial Doppler (TCD) has steadfastly achieved an important diagnostic niche in the diagnosis and management of a large variety of cerebrovascular disorders. TCD applications almost exclusively center on examining the basal cerebral arteries (BCAs) and several of the tributaries that comprise the circle of Willis (Fig. 1). The unifying principles comprise a low-intensity, focused, pulsed acoustic wave with frequency on the order of 1–2 MHz, aimed toward the location of desired vessel. Successful insonation provides vessel depth and continuous flow velocity information for the blood flow within the insonated portion of the vessel. This Doppler-derived data is converted into audible and visual signals to guide the technician to achieve the maximum signal intensity and thereby a representation of the aggregate velocities at that point within the vessel. Visual imaging of the entire spectra is encapsulated into flow velocity envelopes that mirror each cardiac cycle. Changing the location of the probe results in interrogation of adjacent vessel territories; angling the probe in the direction of the vessel and changing beam depth of focus allows the technician to insonate all of the vessels that compromise the circle of Willis.

TCD has yet to be superseded by other more elaborate techniques. Despite marked advances in other neuroimaging techniques such as magnetic resonance imaging (MRI) with diffusion-weighted scanning, computed tomography, and MRI angiography in helping to decipher the impact of vascular disorders on cerebral perfusion and related injury mechanisms, TCD remains an increasingly important neuroimaging adjunct because of its high temporal resolution and easy portability to the bedside of critically ill patients with severe neurologic injury. The technique also proves valuable real-time continuous evaluation of flow characteristics and velocity patterns during dynamic clinical conditions where perturbations in cerebral blood flow (CBF) can be severe and rapid correction with medical treatment is necessary. Since the technique provides for continuous visual inspection, mapping the circle of Willis can provide data on CBF disturbances produced by, e.g., vasospasm, vascular stenosis, compensatory flow patterns, and hyperperfusion. Currently, TCD is the only technique that is able to detect emboli as they transit through the blood vessel.

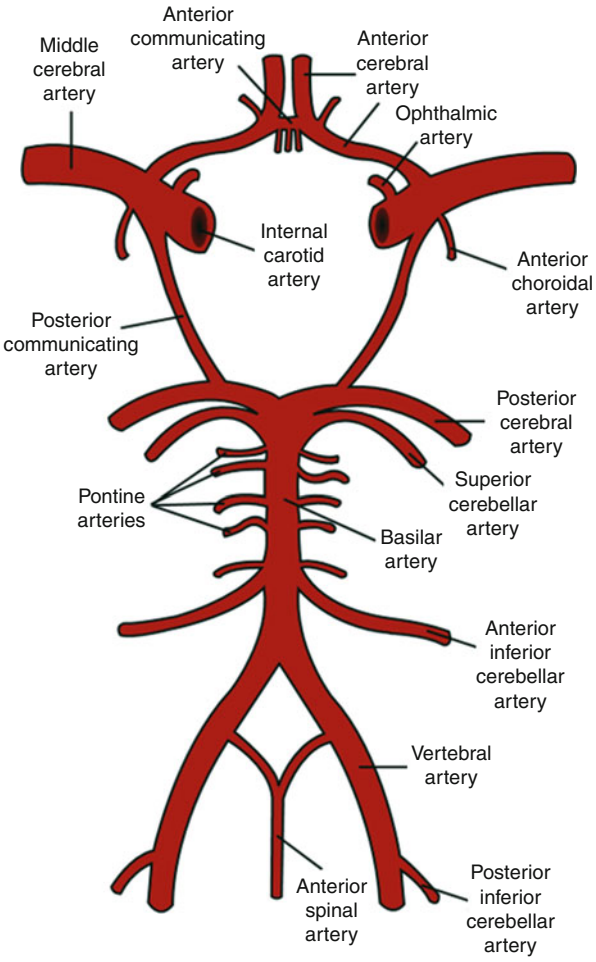

Fig. 1 The Circle of Willis is the central vascular hub within the cerebral arterial circulation. Blood is supplied by the basilar artery and internal carotid arteries and delivered to the Circle of Willis, where several vessels branch off to supply blood throughout the brain. Of note, the middle cerebral artery, anterior cerebral artery, and posterior artery branch off the Circle of Willis to carry blood to their perfusion territories. Image is public domain

This chapter reviews the physical mechanisms of the TCD modality, followed by current clinical indications. Some rapidly evolving usages, especially in the fields of early-onset disease diagnosis and neurocognitive assessment, are discussed at the end of the chapter.

Physical Principles

Sound Generation

This section provides an overview of the physical principles used in transcranial Doppler; for a more detailed treatment, refer to ▶ Chap. 44, "Ultrasonic Measurement of Blood Flow Velocity and Applications for Cardiovascular Assessments" in this

volume. TCD uses similar physical principles that apply to all acoustic imaging systems. Ultrasound uses mechanical wave propagation that is above the audible range (>20,000 Hz). Ultrasound instrumentation utilizes the piezoelectric effect whereby electrical energy is converted to an oscillating ultrasonic wave through expansion and contraction of a piezoelectric crystal due to cyclical changes in polarity of the electrical impulse. The ultrasound frequency is fixed by the physical features of the piezoelectric crystal, and for TCD, this frequency may range from 2 MHz in adult examinations (Kumar and Alexandrov 2015) to 5–10 MHz in infants (American Institute of Ultrasound in Medicine 2012) and even 16 MHz in neurosurgical applications. Commercial instrumentation can focus the Doppler beam at the desired distance as well as control the range of acquisition ("sample volume" or "gate") at the location of its intended vascular target. Reflected ultrasound energy returns back to the piezoelectric element, exerting mechanical force on the crystal, which produces voltage proportional to the mechanical forces exerted on the crystal by ultrasonic energy.

Measurement of Doppler Spectrum

Transcranial Doppler ultrasound operates on the principles of pulsed-wave Doppler ultrasound. In pulsed-wave Doppler, a very short pulse of mechanical sound energy is emitted by the transducer. The pulse of sound is reflected off of small scatterers in the blood vessel being insonated, such as red blood cells, and returns to the transducer, where it is detected. The velocity of the small scatterers in the blood has an effect on the time it takes for subsequent pulses to return to the transducer. By measuring the change in return time from one pulse to the next, the velocity of scatterers in the blood can be estimated via the Doppler equation. The velocity versus time display in TCD contains a range of velocities at every moment in time, representing the range of velocities present in the insonated artery. The TCD waveform, often referred to as the **TCD envelope**, is defined as a wave that traces the maximum velocity present in the velocity versus time display at any moment in time (Fig. 2). Other blood flow velocity parameters may be measured from the TCD envelope, including **systolic velocity** (V_s, the maximum velocity present in the envelope waveform over one heartbeat cycle), **diastolic velocity** (V_d, the minimum velocity present in the envelope waveform over one heartbeat cycle), **mean velocity** (V_m, the average of the envelope waveform over one heartbeat cycle), Gosling's **pulsatility index** ($PI = (V_s - C_d)/V_m$) (Gosling and King 1974), and Pourcelot's **resistivity index** ($RI = (V_s - V_d)/V_s$, (Petersen et al. 1997). The significance of PI and RI, as well as V_s, V_d, and V_m, will be discussed below under "Interpreting Doppler Spectra."

Other Considerations: Aliasing, Sample Volume, and Doppler Angle

Due to the principles of pulsed-wave Doppler, several conditions are required to ensure accurate TCD spectra. The first condition is that the pulse repetition frequency (PRF) is high enough to avoid "aliasing," which occurs when blood flow

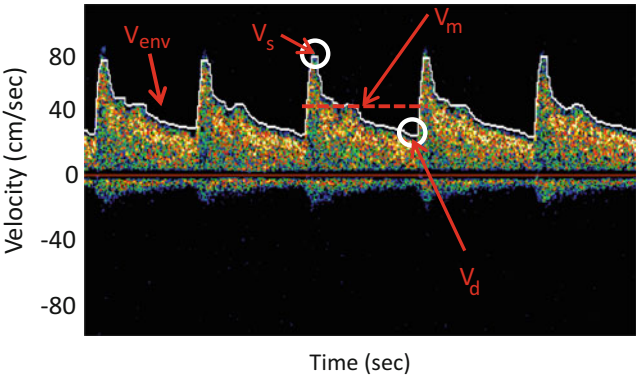

Fig. 2 Example TCD output with the spectral flow envelope (V_{env}) shown outlined in white. Note the pulsatile nature of the blood flow velocity versus time. Systolic velocity (V_s), diastolic velocity (V_d) and mean velocity (V_m) are marked for one heartbeat cycle

velocities are measured which are not actually present in the artery. This can be avoided by increasing the PRF or decreasing the maximum velocity present in the velocity versus time display. A second condition is that the sample volume should be small enough to ensure that only the artery of interest is being insonated; too large of a sample volume can cause the TCD spectra from several adjacent arteries to be displayed as one spectrum. It is usually best to start with a large sample volume when initially searching for arteries and then decrease the sample volume once the desired signal is found. Finally, it is important to remember that due to the Doppler equation, the velocities displayed in the velocity versus time spectrum will only be accurate up to a factor of cos(θ) (Deppe et al. 2004). Therefore, the actual velocity present in the artery will always be greater than or equal to the velocity displayed on the TCD machine, and the amount of difference will depend on the transducer's position and angle relative to the artery being insonated.

Factors Affecting Signal Strength

Other factors that are important in generating spectra that accurately reflect the flow metrics through the insonated vessel include intensity, impedance, and attenuation. Intensity is defined as the ultrasound energy flux measured in watts over the cross-sectional area of the beam. In TCD applications, power adjustment is necessary to achieve penetration through the insonation site to achieve adequate return signal strength. Factors that affect intensity include beam width, depth of insonation, and tissue density. The presence or absence of bone at the site of insonation proves to be a chief factor determining the acoustic intensity necessary to obtain adequate signal. As little as 5–10 mW/cm^2 is required to achieve an adequate spectral examination through the anterior fontanelle, foramen magnum, or orbit, whereas 50–200 mW/cm^2 is required through the transtemporal window in most children and adults. Increased

bone density such as hyperossification in patients with sickle cell disease may require intensity as high as 700 mW/cm^2. In clinical practice, acoustic transmission also requires ensuring a suitable probe-tissue interface. Failure to use a suitable acoustic liquid gel to eliminate air and ensure a smooth interface can lead to marked attenuation, reflection, and scattering of the acoustic energy by more than 99 %, precluding an effective examination.

Safety

Although ultrasound is a very safe imaging modality, it is good practice to limit patient exposure, both in terms of the power used and insonation time (American Institute of Ultrasound in Medicine 2012). When conducting TCD examinations, some clinicians will initially set power to a high value; this shortens the time needed to find the blood vessel of interest and can therefore decrease the overall dose of mechanical energy received. However, once the vessel is found, power is immediately reduced to the minimum amount needed to achieve a usable signal in accordance with the ALARA (As Low As Reasonably Achievable) principle (Wells 2006).

Interpreting Doppler Spectra

Training is necessary to interpret TCD spectra. Information of clinical use may be obtained from the velocity parameters (defined above) such as V_s, V_d, V_m, the pulsatility, and the resistivity indices, as well as from other characteristics of the TCD envelope. The shape of the envelope provides information about resistance distal to the point being insonated and, along with direction of flow, the depth of the insonated region, and velocity parameters such as V_m, PI, and RI, allows artery identification (for a list of normal velocity values, PI values, and depths, see (Alexandrov and Neumyer 2004). A large difference between V_s and V_d indicates high distal resistance, and a small difference between V_s and V_d indicates low resistance. The pulsatility index (PI) and resistivity index (RI) also capture information about resistance distal to the point being insonated (Petersen et al. 1997; Naqvi et al. 2013). Normal values for the PI are 0.5–1.19, with values less than 0.5 indicating distal vasodilation and values greater than 1.19 indicating distal vasoconstriction or occlusion; normal values for the RI are slightly less than for the PI, with values $>$0.8 indicating distal vasoconstriction or occlusion (Naqvi et al. 2013).

Changes in cerebral blood flow velocity are well-correlated with changes in CBF, as long as the diameter of the vessel being insonated does not change significantly, which is often the case for the basal cerebral arteries (Deppe et al. 2004).

The direction of flow (toward or away from the transducer) allows artery identification and may also allow detection of abnormalities, such as a steal (will show reversal of blood flow, as blood intended for one vessel is instead sent to a different vessel) or a totally occluded artery (low but equal velocities in opposite directions during systole and diastole). Typical Doppler spectra for several basal arteries in

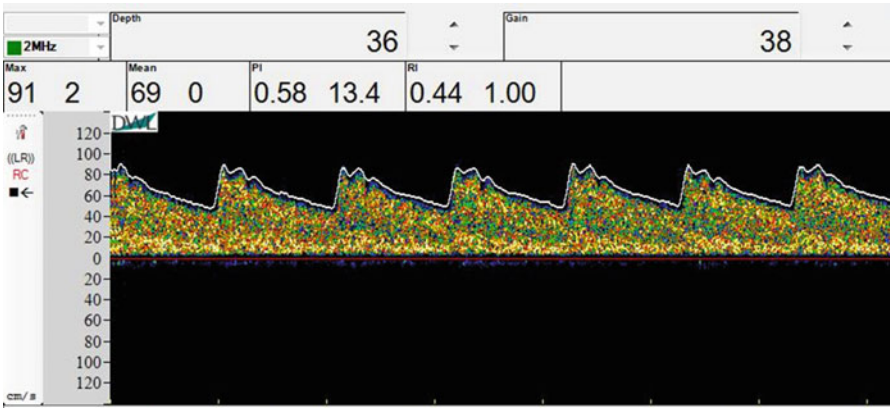

Fig. 3 Healthy blood flow through the distal middle cerebral artery is visible in the TCD spectra. Note the relatively shallow insonation depth of 36 mm

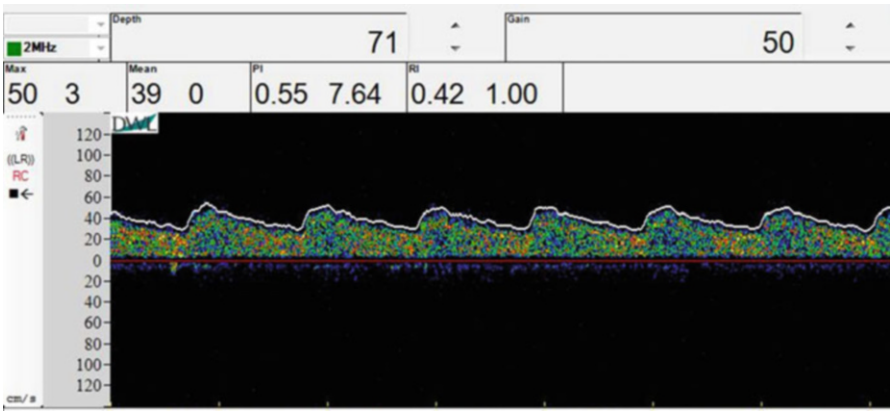

Fig. 4 TCD spectrum of posterior cerebral artery, showing low-resistance flow. Note the lower maximum and mean velocities than are present in the middle cerebral artery (the first numbers under the headings "max," "mean," "PI," and "RI" refer to flow towards the transducer); also note the depth of 71 mm

healthy subjects are shown in Figs. 3, 4, 5, 6, 7, and 8. These spectra demonstrate the differences in the hemodynamic parameters described above that are seen within the normal brain.

Current Clinical Use

The most common method of assessing the circle of Willis is using a handheld method where a well-trained technician controls power amplitude, depth of insonation, sample volume, and the angle of insonation. Technicians must have

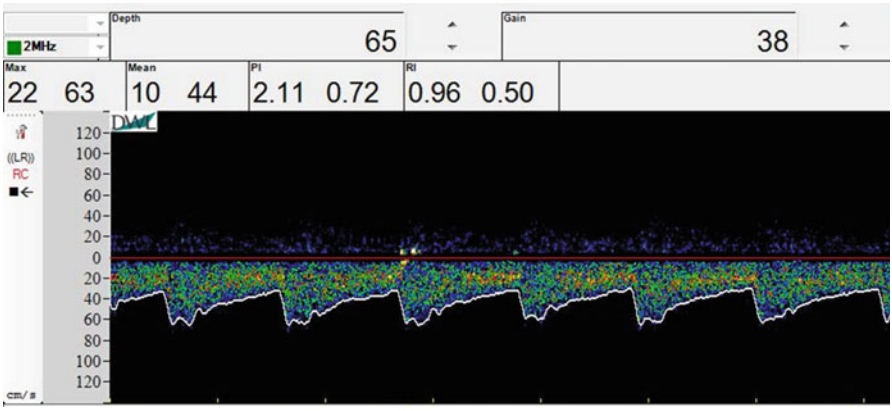

Fig. 5 TCD spectrum of anterior cerebral artery (ACA). Note the retrograde flow (towards the transducer, below the *red baseline*). The second values under the headings of maximum velocity, mean velocity, pulsatility index (PI), and resistivity index (RI) refer to blood flow towards the transducer

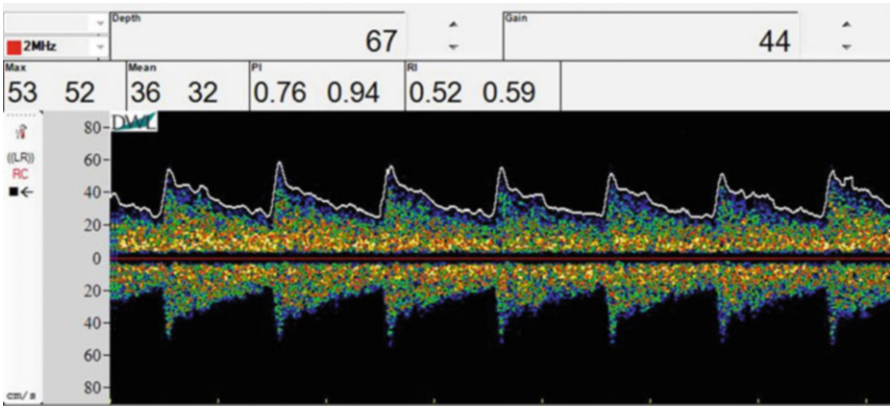

Fig. 6 TCD spectrum of the bifurcation of the anterior cerebral artery (ACA) and middle cerebral artery (MCA), showing both anterograde (towards the transducer) and retrograde (away from the transducer) flow at the same depth. The bifurcation is a landmark used in TCD examinations. When locating the bifurcation, the depth and angle should be adjusted until the signal with the brightest spectrum in both directions is found

considerable knowledge of the anatomy and variation of the circle of Willis, vessel depth in relation to the insonation window, vessels that can be interrogated via each of the windows, and creation of suitable visual displays of the spectral analysis of the reflected acoustic data. Technicians should have extensive experience in assessing the circle of Willis when assessing each of the BCAs.

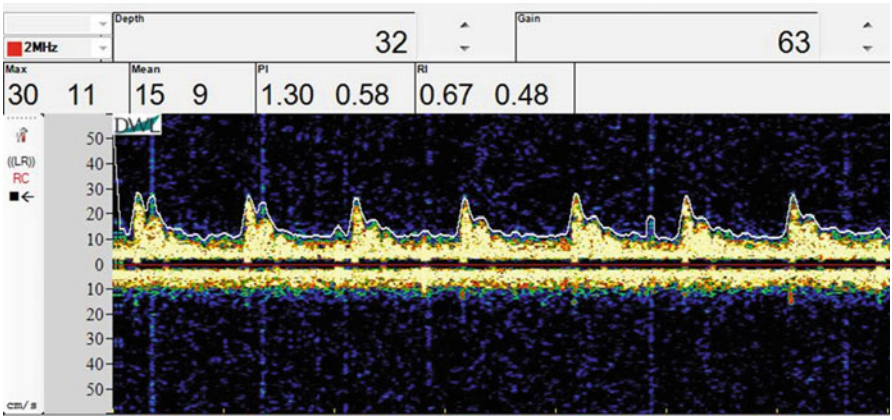

Fig. 7 TCD spectra of the ophthalmic artery. Note the elevated PI and RI values, indicating higher resistance. Additionally, note the distinctly increased flow velocity during systole as compared to diastole. Greater visibility of background noise due was caused by applying a high gain to obtain an optimal flow envelope

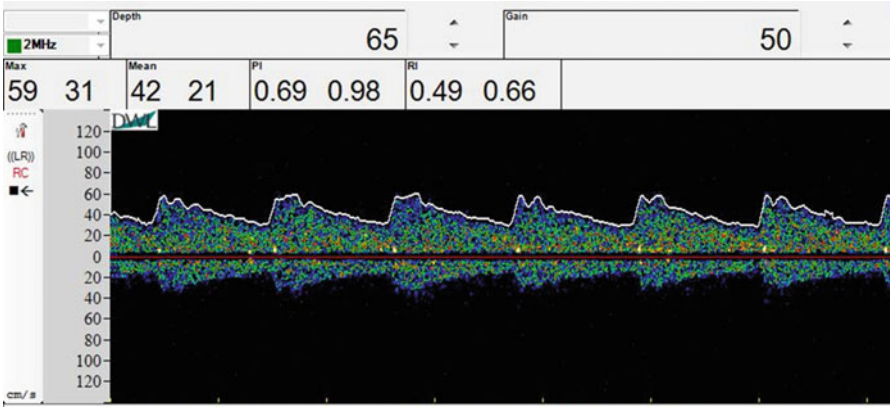

Fig. 8 TCD spectrum of the internal carotid artery, indicating both positive and negative flow velocities, suggesting blood flow in two directions. Low resistance flow is shown by the relatively small difference between the systolic velocity and the diastolic velocity

Emboli

As early as 1998, there was consensus that emboli detection via TCD is important (Ringelstein et al. 1998), and several studies have confirmed negative neurological outcomes in adults are correlated with emboli (e.g., Stump et al. 1999). Tragically, brain injury (both short-term and long-term) is the most common complication of cardiac surgery in pediatric patients (Hirsch et al. 2012; Su and Undar 2010). Its cause is

hypothesized to involve many factors, including alterations in cerebral flow and metabolism that lead to poor oxygenation of tissue and cell death. While many of these factors have been studied, the role of cerebral emboli generated by cardiopulmonary bypass and the surgical procedure is poorly understood, especially in infants and children. It is known that during cardiac surgery, emboli, which include tissue fragments, air bubbles, platelet thrombi, fibrin plugs, or microscopic flakes of catheters and vascular tubing, are inadvertently introduced into the systemic circulation. It is also known that once emboli enter the bloodstream, they can move into the brain and create dangerous blockages of cerebral vessels that lead to neurological damage.

TCD is an ideal modality for monitoring emboli passage through blood vessels because of the sharp echo signature emboli produce in the Doppler spectrum. The signature is clearly identifiable both visually and aurally, producing audible "chirps" that give real-time feedback to clinicians of timing and number of emboli. Research continues for robust algorithms for discrimination of emboli (between gaseous and particulate), as well as sizing of emboli.

Traumatic Brain Injury

Traumatic brain injury (TBI) is a growing medical concern, affecting approximately 1.7 million people in the United States every year (Centers for Disease Control and Prevention 2010). Nearly 80 % of TBI patients are seen in a hospital emergency room, resulting in approximately 275,000 hospitalizations and over 50,000 deaths each year. Tragically, an estimated 5.3 million Americans live with disabilities resulting from traumatic brain injury. Patients with severe traumatic brain injury have a significant risk of hemorrhage and cerebral edema.

A subgroup of these patients will develop associated bleeding complications including epidural, subdural, intracerebral, and subarachnoid hemorrhage (SAH), which can induce a secondary brain injury from ischemia or infarction within a vascular territory (stroke). The mechanism of injury is associated when a damaged vessel bleeds into the space between the arachnoid membrane and the pia mater. The same event can also occur when a developmental anomaly within the vascular wall (aneurysm) ruptures and blood extravasates within the same space. Visible bleeding detected by neuroimaging may not be identified in up to 15 % of cases (Raya and Diringer 2014). The extravascular blood produces a secondary injury to the blood vessel that results in varying levels of cerebral vasospasm, which causes constriction of the affected blood vessel and restricting of CBF in the distal cerebral territory fed by the blood vessel. The vasospasm is often delayed from 3 to 14 days after the bleeding event. Hemorrhage-associated vasospasm is capable of producing progressive decline in neurologic function, coma extensive cerebral infarction, and sometimes brain death (Rigamonti et al. 2008). Currently, the gold standard for vasospasm diagnosis is cerebral angiography. However, this procedure does not allow for bedside monitoring, which is needed for unstable critically ill patients with

vasospasm SAH. Recently, TCD has emerged as an inexpensive, noninvasive tool used for bedside monitoring of vasospasm after SAH (Marshall et al. 2010). Close monitoring of patients with TCD following SAH permits early treatment of the development of narrowing cerebral arteries through medication.

Cerebral edema is swelling in parenchymal brain tissue due to increased intracranial pressure from fluid accumulation as a result of a severe TBI or any nontraumatic ischemic event. It is a potential cause of the devastating consequence of cerebral herniation, which if not quickly corrected leads to either a vegetative state or death (Asil et al. 2003; Arch and Sheth 2014). TCD may be used in two situations for detection of cerebral swelling: in its conventional way, measuring blood flow in cerebral arteries, looking for decreased flow as a result of pressure from edema in the territories being fed by the insonated blood vessels, and also as a monitor of intracranial pressure, which is related to hemodynamic indices. TCD studies have shown a correlation between PI and cerebral edema (Muttagin et al. 1993). Cerebral swelling from direct brain injury typically occurs within the first 1–3 days after the inciting event, far earlier than witnessed with brain injury as a result of cerebral vasospasm. Rising resistance indices within one or more vessels during the time frame of the two mechanisms of injury is useful in detecting these two vascular complications, and necessary interventional therapies can be delivered to ameliorate or reverse permanent cerebral damage that can ensue.

Sickle Cell Disease

Sickle cell disease (SCD) is a life-threatening genetic disorder that affects nearly 100,000 individuals in the United States (Yawn et al. 2014) and is associated with life-threatening cerebral complications. Cerebral complications primarily result from progressive vascular stenosis principally located at the juncture of the intracranial component of the internal carotid artery, middle cerebral artery, and anterior cerebral artery. Progressive stenosis results in a gradual attenuation of CBF to the distribution of the anterior circulatory territories that comprise more that 80–85 % of the cerebral hemispheres. A subset of the SCD population develop a particularly aggressive course and can suffer cerebral infarction as early as 4 years of age and continue to suffer new cerebral infarction as the disease progresses. The primary therapy to control disease progression is long-term blood transfusions to reduce the sickle cell percentage of the blood to less than 30 % (Yawn et al. 2014). In order to avoid wasting valuable blood resources and exposing the entire SCD population to repeated blood transfusions, screening for stenosis is the ideal method to identify the population at risk for stroke. TCD can identify with high-fidelity children with sickle cell disease who develop a risk of stroke (Adams et al. 1992). Annual TCD monitoring with intervention in cases with high TCD velocities (more than 200 cm/s) is the standard of care in children with sickle cell disease and has been shown to effectively reduce the incidence of stroke (Sloan et al. 2004).

Cardiac Right-to-Left Shunts

Patent foramen ovale is a relatively common residual congenital cardiac defect with an incidence of about 5 % in the general population (Wu et al. 2004). The presence of an intracardiac shunt has long been considered an emerging cause of cardioembolic cerebral infarction (Arboix and Alio 2012). It is unclear whether or not it is causal to stroke or transient ischemic attack (Katsanos et al. 2014). TCD has been used to detect with a high degree of fidelity right-to-left shunts (RLS) such as patent foramen ovale, atrial septal defect, and patent ductus arteriosus. A meta-analysis showed weighted mean sensitivity and specificity of 97 % and 93 %, respectively, when using TCD to detect RLS, using transesophageal echocardiography (TEE) as the standard (Mojadidi et al. 2014), and a preliminary study showed that, when TCD and TEE or transthoracic echocardiography were both performed, 25 % of the population TCD detected an RLS that the other method did not (de Havenon et al. 2015).

Brain Death

The concept of brain death was first introduced in 1959, marked as the "irreversible cessation of all functions of the entire brain" (Wijdicks 2001). According to the most recent report of the Quality Standards Subcommittee of the American Academy of Neurology (Wijdicks et al. 2010), in addition to cerebral angiography and electroencephalography (EEG), TCD is listed as a method of ancillary testing for the determination of brain death (Wijdicks et al. 2010). Abnormalities that may be identified in the MCA suggesting the event of brain death include reverberating flow or small systolic peaks during early systole (Wijdicks et al. 2010). The main advantage of TCD in comparison to EEG or cerebral angiography is the diagnostic power for early confirmation of brain death. Early confirmation of brain death gives families time to cope and reach closure and improves the opportunity to consider organ donation.

Potential Applications to Prognosis and Other Diseases or Conditions

Functional TCD

The relationship between neural activity and cerebral blood flow has long been known since first described by Fulton (1928). Later studies have confirmed a close relationship between brain activity and blood flow (Raichle et al. 1975; Heiss and Podreka 1978; Kuschinsky 1991). Cerebral blood flow (CBF) is regulated by the vasodilation and vasoconstriction of small cerebral arteries (Huber and Handa 1967) and cerebral precapillaries and arterioles (Itoh and Suzuki 2012). Currently, functional magnetic resonance imaging (fMRI) is a popular technique used to measure hemodynamic changes that can be related to neural activation (e.g., see (Hurschler

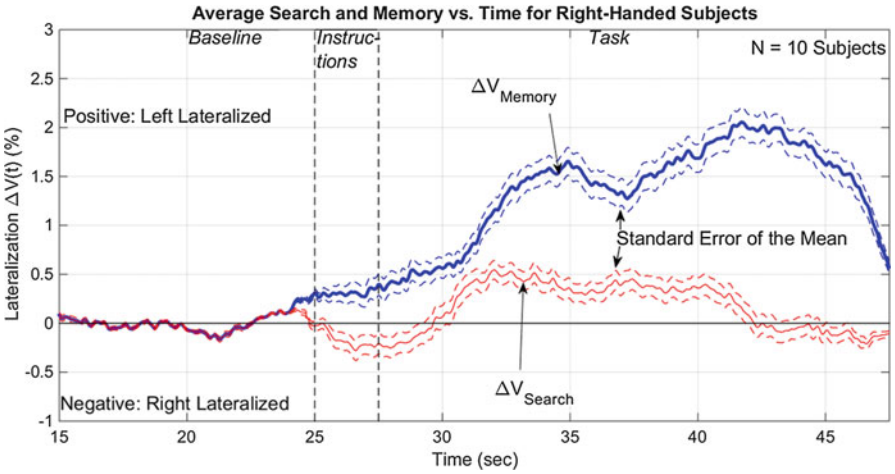

Fig. 9 Example of the use of functional TCD to measure the lateralization in response to two different stimuli: a search task and a memory task. The average lateralization versus time for the two tasks ($\Delta V_{Search}(t)$ and $\Delta V_{Memory}(t)$) is shown by *solid lines*. *Dashed lines* above and below *solid lines* represent +/− 1 standard error of the mean. The experiment consisted of a baseline period in which no stimuli were presented, an instruction period in which subjects had to read instructions, and a task period in which subjects were shown a visual scene on a computer screen and asked to perform the appropriate task (Hage et al. 2015)

et al. 2015; Greve et al. 2013; Poldrack 2012), but this technique has the disadvantages of high cost and having limited time resolution for imaging transient changes in hemodynamics (Marxen et al. 2012).

After describing TCD in 1982, Aaslid was able to show an increase in CBFV in the posterior cerebral artery in response to a visual stimulus (Aaslid 1987), one of the first demonstrations of "functional" TCD (fTCD). One important application of fTCD was in determining hemisphere dominance, eventually replacing the Wada test, which is an invasive test administered before epilepsy surgery in order to determine the dominant hemisphere for language in a patient (Knecht et al. 1998). Studies have also shown that fTCD can provide very accurate information on neural activation and lateralization during cognitive tasks; for example, on verbal tasks (Knecht et al. 1996, 1998; Meyer et al. 2014; Deppe et al. 2004; Vingerhoets and Stroobant 1999), visuospatial tasks such as design comparison and mental rotation of figures (Vingerhoets and Stroobant 1999), and perceptual speed and visual discrimination tasks (Schmidt et al. 1999). Most recently, studies have shown the ability of TCD to determine lateralization (Fig. 9) in visual memory and visual search tasks simultaneously (Hage et al. 2015). Some studies have shown right lateralization of blood flow in the MCA during emotional responses to negative stimuli, suggesting that the right hemisphere is more active in processing emotional stimuli than the left; it has also been shown that this right lateralization in response to emotional stimuli is absent in Parkinson's disease patients (Troisi et al. 1999, 2002). Future work may extend this study of lateralization in the processing of emotions to depressed patients.

Alzheimer's Disease

Currently, dementia affects more than 44 million people worldwide (Alzheimer's Disease International 2014). Alzheimer's disease (AD) is the leading cause of dementia, where approximately 50–75 % of all dementia cases are caused by AD. There is no single test to diagnose AD. However, a variety of approaches and tools are available to help make a diagnosis (Alzheimer's Association 2015). Current diagnostic tools and measures are either invasive (cerebrospinal fluid (CSF) proteins) or expensive (Pittsburgh compound B (PIB) brain scan) (Laske et al. 2015). TCD is a noninvasive, cost-effective tool, and studies have shown that TCD may be a very promising screening tool for AD. CBFV, pulsatility index (PI), and cerebrovascular reactivity (CVR) are the most studied parameters for AD diagnosis with TCD (Tomek et al. 2014).

Compared with healthy control subjects, research using TCD suggests that individuals with AD and mild cognitive impairment (MCI) have significantly lower CBFV and higher PI values, particularly in the middle cerebral artery (Stefani et al. 2009). Moreover, a longitudinal study showed that subjects with greater CBFV velocity were less likely to develop Alzheimer's disease (Ruitenberg et al. 2005).

CVR is a change in CBVF in response to a vasodilatory or vasoconstrictive stimulus (Fierstra et al. 2013). The primary types of vasoactive stimuli include the injection of acetazolamide (Markus and Harrison 1992), the breathing of a 5 % carbon dioxide gas mixture, and the breath-holding method. The most commonly used stimulus utilized to measure CVR with transcranial Doppler is the breath-holding (BH) test. In this method, CVR is calculated by the following formula (Shim et al. 2014)

$$\frac{(\text{Average CBFV before BH}) - (\text{maximum CBFV during BH})}{\text{average CBFV before BH}} \times 100\% \quad (1)$$

Studies indicate not only significantly lower CVR values in AD and MCI patients versus controls but additionally show significantly lower CVR values in AD versus MCI (Shim et al. 2014). This method is accurate enough to distinguish between AD and MCI. This accuracy may be used for future applications in distinguishing the preclinical stage of Alzheimer's disease.

Mild Traumatic Brain Injury and Concussion

Concussion (a subset of TBI) affects hundreds of thousands of high school, college, and professional athletes each year (Len et al. 2011; Alsalaheen et al. 2010). An estimated 300,000 sports-related concussions occur annually in high school alone (Marar et al. 2012). Concussions are a complex pathophysiological process that can have long-term consequences on an athlete's brain function, balance, and behavior (McCrory et al. 2013).

Some studies suggest that normal CVR responses may be disrupted immediately after concussion (Len et al. 2011; Dewitt and Prough 2003). TCD provides a useful method for assessing CVR impairment after concussion. Combining transcranial Doppler technology with standard postural control feedback measurement methods could offer an objective, quantitative tool for clinical use as an improved concussion-screening protocol. The ability to simultaneously and synchronously acquire cerebral hemodynamic data and postural control data has recently been shown (Honaker et al. 2015).

Summary Points

- Transcranial Doppler is a subset of the conventional diagnostic ultrasound modality that is specifically designed to measure blood flow in cerebral arteries.
- Transcranial Doppler is equivalent to pulsed-wave (PW) Doppler in conventional ultrasound, operating at a low frequency (1–2 MHz) in order to penetrate the cranium.
- Compared to other brain-imaging modalities, TCD is inexpensive, easily portable, and provides real-time (on the order of 100 samples per second) blood flow velocity data.
- The dynamic nature of transcranial Doppler, and the ease of capturing fast transient blood flow responses due to artificial stimuli, allow for diagnosis of a variety of pathologies affecting cerebral autoregulation.
- Because blood flow has been shown to be correlated with neural function, TCD can be used in a variety of ways to locate and quantitate brain activity.

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Epicardial Fat Thickness as a Biomarker in Cardiovascular Disease

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Gianluca Iacobellis

Contents

Abstract

Epicardial adipose tissue is the visceral fat depot of the heart with unique anatomical and functional properties. Epicardial fat can be considered a novel biomarker of cardiovascular disease. Its thickness can be visualized and measured using standard two-dimensional echocardiography with several advantages, including its low cost, easy accessibility, and good reproducibility. Echocardiographic epicardial fat thickness reflects the intra-abdominal visceral fat and

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_13

intramyocardial fat accumulation. Epicardial fat thickness is related to traditional and novel cardiovascular risk factors. Epicardial fat thickness correlates and predicts the risk of metabolic syndrome. Epicardial fat has been associated with the presence and severity of coronary artery disease, independent of traditional cardiometabolic risk factor and coronary calcification. Given its rapid metabolism and its simple objective measurability, epicardial fat can serve as target for pharmaceutical agents targeting the adipose tissue.

Keywords
Epicardial fat • Epicardial adipose tissue • Biomarkers • Visceral fat • Echocardiography

Key Facts of Epicardial Fat

- Epicardial fat is the visceral fat depot of the heart with unique features.
- Epicardial fat has physiological and pathological properties.
- Epicardial fat can be easily measured with imaging techniques.
- Epicardial fat is a marker of visceral fat and cardiovascular risk.
- Epicardial fat thickness is a therapeutic target.

Introduction

Human body fat is functionally heterogeneous and not equally distributed. Local fat accumulation seems to be more important than overall body fat. If increased visceral fat is considered a major determinant of a poor cardiometabolic profile, the excessive intraorgan fat is recently thought to play a key role in cardiovascular diseases. Consistently with the emerging concept of organ-specific adiposity, I focused my attention on the epicardial fat, the visceral fat depot of the heart.

Anatomy and Embryology of the Epicardial Fat

The adipose tissue of the heart is divided into two layers: epicardial fat, the visceral layer, and pericardial fat, situated externally to the parietal layer of the pericardium (Iacobellis 2005, 2009). Epicardial and intra-abdominal fat evolve from brown adipose tissue during embryogenesis (Marchington et al. 1989). Epicardial fat is supplied by branches of the coronary arteries. In the adult human heart, epicardial fat is commonly found in the atrioventricular and interventricular grooves. As the amount of epicardial fat increases, it progressively fills the space between the ventricles, sometimes covering the entire epicardial surface. Notably, no muscle fascia divides epicardial fat and myocardium; therefore, the two tissues share the

same microcirculation (Iacobellis 2005). This allows the hypothesis of a direct interaction between the epicardial fat and the myocardium.

Physiological and Biochemical Properties of the Epicardial Fat

A dichotomous role, both unfavorable and protective, has been attributed to epicardial fat, but its physiology in animals and humans is not completely clear (Iacobellis 2011).

Under normal physiological conditions epicardial fat could therefore serve several distinct functions – as a buffer, absorbing fatty acids and protecting the heart against high fatty acid levels, as a lipid storage and local energy source at times of high demand, channeling fatty acids to the myocardium, and perhaps as brown fat to defend the myocardium against hypothermia (Marchington and Pond 1990; Sacks et al. 2009; Pezeshkian et al. 2009). The brown fat properties of the epicardial fat are not fully elucidated and object of recent investigations (Sacks et al. 2013). Under pathological circumstances epicardial fat releases factors that promote harmful coronary artery and myocardial changes. A body of evidence shows that epicardial fat is an extremely active organ that produces several bioactive adipokines with both proinflammatory and anti-inflammatory properties (Mazurek et al. 2003). Nevertheless, what could influence this equilibrium between harmful and possible protective effects is still unknown. Because of its anatomical proximity to the heart and the absence of fascial boundaries, epicardial adipose tissue may interact locally and modulate the myocardium and coronary arteries through paracrine or vasocrine secretion of proinflammatory adipokines (Iacobellis 2011; Sacks 2007) (Fig. 1).

Echocardiographic Epicardial Fat Thickness

Epicardial fat thickness can be visualized and measured with two-dimensional guided M-mode echocardiography using commercially available equipments, as first proposed and validated by Iacobellis (Iacobellis 2003, 2009) (Fig. 3). Standard parasternal long- and short-axis views permit the most accurate measurement of epicardial fat thickness on the right ventricle. Echocardiographically, epicardial fat is generally identified as the echo-free space between the outer wall of the myocardium and the visceral layer of pericardium and its thickness is measured perpendicularly on the free wall of the right ventricle at end-systole in three cardiac cycles. The majority of population-based clinical studies have reported excellent interobserver and intraobserver agreement on epicardial fat thickness measurement. Echocardiographic epicardial fat thickness range varies from a minimum of 1 mm to a maximum measured value of almost 25 mm. The wide range of epicardial fat thickness likely reflects the substantial variation in abdominal visceral fat distribution (Iacobellis 2003).

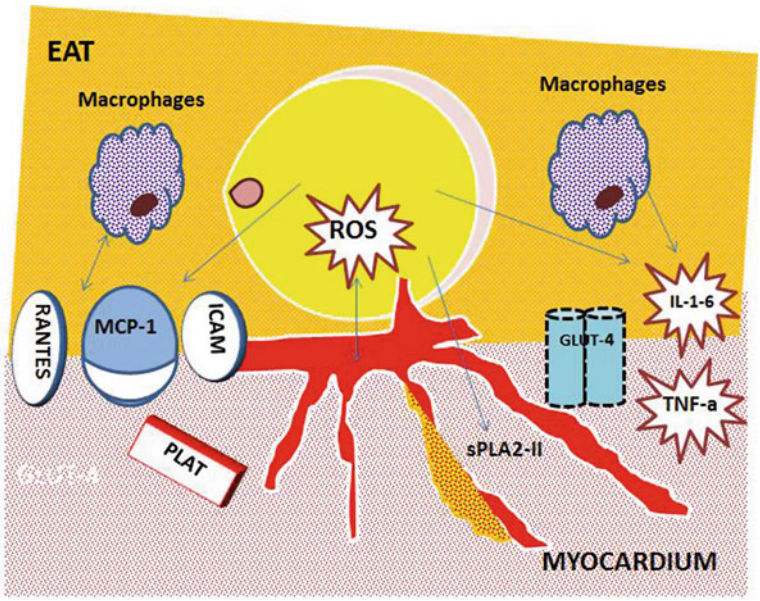

Fig. 1 Paracrine pathological effects of epicardial fat. Under pathological conditions epicardial adipose tissue (EAT) can locally affect myocardium and coronary arteries by a complex interplay of mechanisms. EAT displays a dense inflammatory infiltrate, mainly represented by macrophages. Both macrophages and EAT adipocytes can release pro-inflammatory and atherogenic cytokines, such as Tumor Necrosis Factor-alpha (TNF-a), Interleukin 1 and 6 (IL1, IL6) and Monocyte Chemoattractant Protein-1 (MCP-1), regulated upon activation t-cell and secreted (RANTES) and soluble intercellular adhesion molecule (ICAM). Increased EAT reactive oxygen species (ROS) also contribute to activate inflammatory signals. EAT Type II secretory phospholipase A2 (sPLA2-II) secretion facilitates lipid accumulation within the atherosclerotic plaque. A local insulin resistance status is also due to the lower EAT glucose transporter-4 (GLUT4) expression. EAT also affect local coagulation and hemostasis by expressing tissue plasminogen activator (PLAT) and other coagulation factors

Epicardial Fat Is a New Marker of Visceral Fat

Echocardiographic epicardial fat is a marker of visceral fat. In fact, echocardiographic epicardial fat strongly reflects the intra-abdominal visceral fat as measured by magnetic resonance imaging and better than waist circumference does (Iacobellis 2003; Iacobellis 2003). In a multiple regression analysis that included waist circumference and epicardial fat thickness, intra-abdominal visceral fat was better and independently predicted by the epicardial fat thickness (Iacobellis 2003) (Fig. 2).

Bland test confirmed the good agreement between the two methods. Other studies confirmed this finding in different populations. Echocardiographic epicardial fat thickness is therefore an independent predictor of visceral adiposity and weakly reflects the obesity degree. Subjects with higher waist circumference clearly show higher epicardial fat thickness, as previously reported. Recent evidences showed that

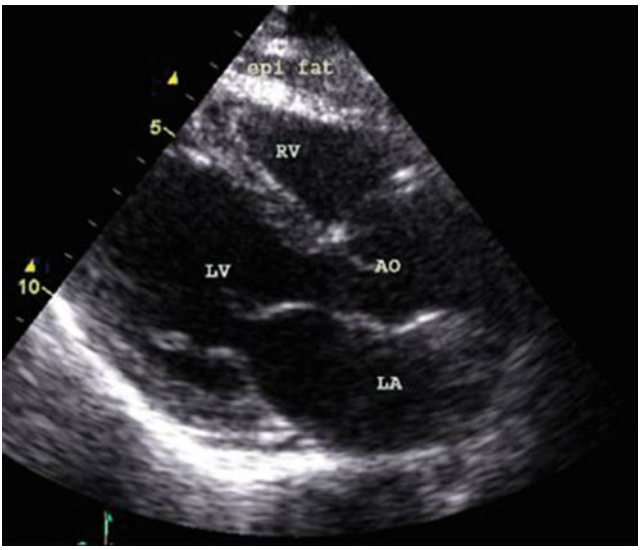

Fig. 2 Echocardiographic **e**picardial fat thickness. Epicardial fat (*epi fat*) is generally identified as the echo-free space between the outer wall of the myocardium and the visceral layer of pericardium and its thickness is measured perpendicularly on the free wall of the right ventricle at end-systole in three cardiac cycles from the parasternal long axis view, as first proposed by Iacobellis

obesity leads not only to increased fat depots in classical adipose tissue locations but also to significant lipid accumulation and infiltration within and around other tissues and internal organs (Iozzo 2011, Kankaanpää et al. 2006). Ectopic fat deposition may occur within the heart and affect cardiovascular function. Myocardial lipid content increases with the degree of adiposity and may contribute to the adverse structural and functional cardiac adaptations seen in obese persons. Echocardiographic epicardial fat is associated with intramyocardial and intrahepatic fat accumulation, as measured by proton magnetic resonance spectroscopy, Malavazos et al. 2010.

Epicardial Fat Is a New Cardiovascular Risk Factor

An escalating number of evidences indicate that epicardial fat measurement may play a role in the stratification and prediction of the cardiometabolic risk. Several clinical studies showed that epicardial fat thickness is also related to traditional and novel cardiovascular risk factors. Epicardial fat thickness is significantly higher in subjects with metabolic syndrome than in those without (Iacobellis 2008; Pierdomenico et al. 2012). When cardiometabolic parameters are considered separately, epicardial fat is independently associated with inflammatory markers, fatty liver, and liver enzymes. A recent meta-analysis reported a significant variability of epicardial fat with ethnicity, with a greater difference in Caucasian subjects than in

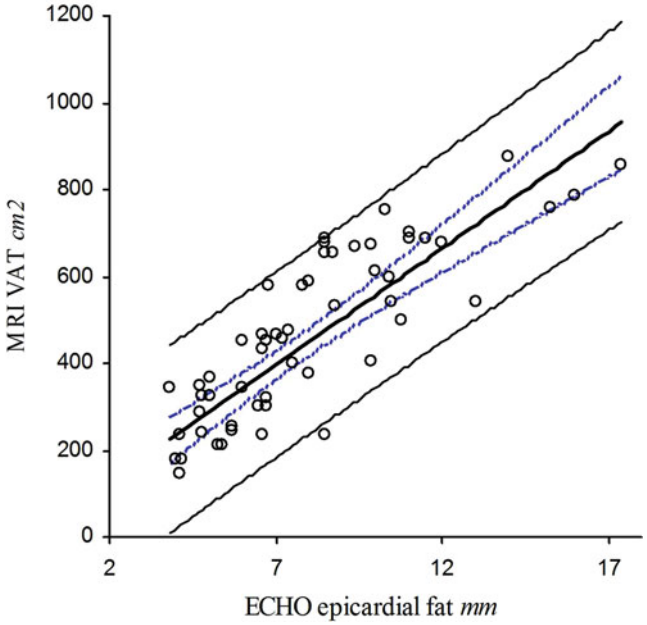

Fig. 3 Echocardiographic epicardial fat thickness as biomarker of visceral fat. Intra-abdominal visceral fat (VAT) area (cm^2), as measured with magnetic resonance imaging (MRI) is independently and significantly correlated with epicardial fat thickness (*mm*), as measured with echocardiography (ECHO)

other ethnic groups, Pierdomenico et al. 2012. Different cutoff points of high-risk epicardial fat thickness for the prediction of metabolic syndrome have been proposed (Iacobellis 2008).

Epicardial fat has been associated with the presence and severity of coronary artery disease in a large number of studies. Epicardial fat contributes to the development and progression of atherosclerosis, independent of traditional cardiometabolic risk factors (Iacobellis 2011) (Fig. 4). The relationship of epicardial fat thickness and coronary artery disease is driven by local mechanisms and is not fully explained by the concurrence of excess visceral fat accumulation or obesity in general. The association of epicardial fat with the risk of coronary artery disease seems to be independent also of coronary calcification. However, further studies to better explain the role of epicardial fat in coronary artery disease would be desirable. Epicardial fat has been also associated with markers of subclinical atherosclerosis, such as carotid intima media thickness, in high-risk individuals. Epicardial fat thickness is also associated with diabetes and insulin resistance in diabetics and no diabetic subjects (Iacobellis 2003, 2008; Momesso et al. 2011). Interestingly, echocardiographic epicardial fat has been reported as the best predictor of ultrasound measured liver steatosis.

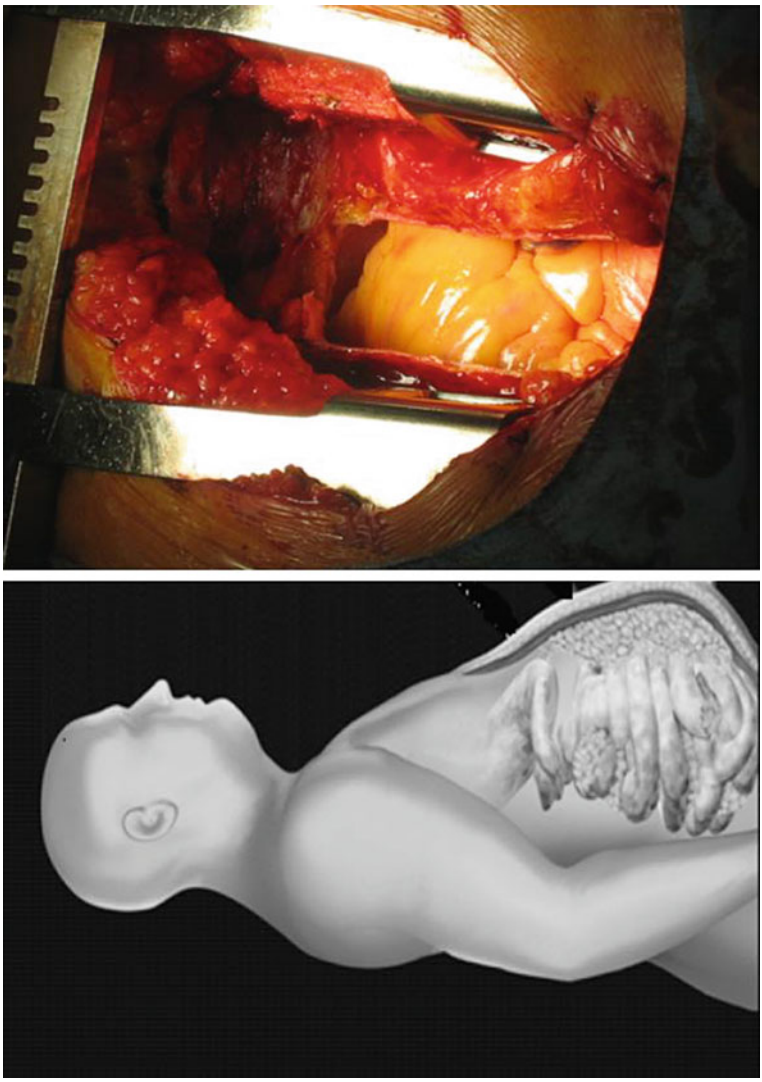

Fig. 4 Epicardial fat as new cardiovascular risk factor. Individuals with excessive intra-abdominal fat accumulation, such as the one depicted here, have large epicardial adipose tissue, as shown in this cardiac surgery picture, and higher risk of developing cardiovascular diseases and major cardiovascular events

Epicardial Fat and Heart Morphology and Function

Increased epicardial fat thickness is related with increased left ventricular mass and abnormal right ventricle geometry, as detected by echocardiography (Iacobellis et al. 2004; Iacobellis 2009). Echocardiographic findings are in agreement with autoptic studies. Mechanical and biomolecular mechanisms have been evoked to explain these correlations. Increased epicardial fat by adding to the mass of the ventricles may increase the work of pumping. Increased left ventricular mass in morbidly obese subjects could be due to a direct effect of excess epicardial fat. Increase in epicardial fat thickness is also significantly correlated with enlarged atria and impaired right and left ventricular diastolic filling in morbidly obese subjects. Epicardial fat may directly contribute to impair diastolic function in subjects with increased visceral adiposity. A mechanical obstacle to diastolic filling due to the excess epicardial fat pad could explain these findings.

Epicardial Fat as New Therapeutic Target

Interestingly, echocardiographic epicardial fat has been reported to significantly and quickly decrease after a very low-calorie diet and bariatric surgery in morbidly obese subjects (Iacobellis 2009; Willens et al. 2007) (Fig. 5). Changes in epicardial fat thickness were significantly higher than changes in BMI and waist circumference after the very low-calorie diet program. Changes in epicardial fat thickness were consensually and independently associated with the improvement in cardiac parameters in these subjects. Given its rapid metabolism and its simple objective measurability, epicardial fat can serve as target for pharmaceutical agents targeting the adipose tissue, such as statins, oral and injectable antidiabetes medications

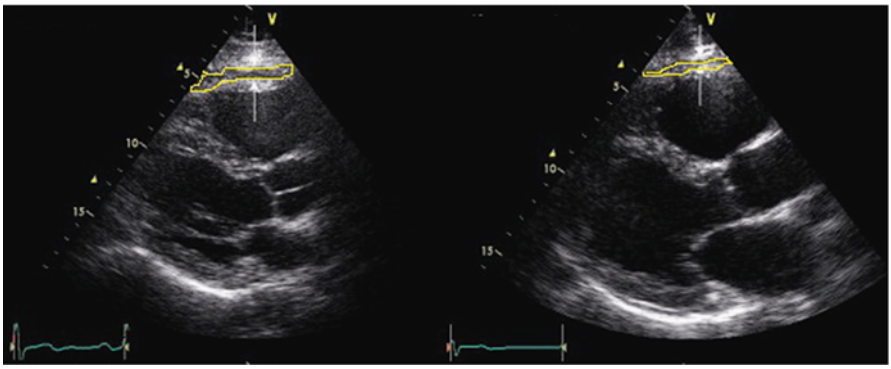

Fig. 5 Echocardiographic epicardial fat thickness as therapeutic target. Echocardiographic epicardial fat can serve as marker of visceral fat changes during pharmaceutical or lifestyle interventions targeting the adipose tissue. These images show a significant and rapid decrease of epicardial fat thickness, within the yellow line, after a 3-month very low calorie diet program.

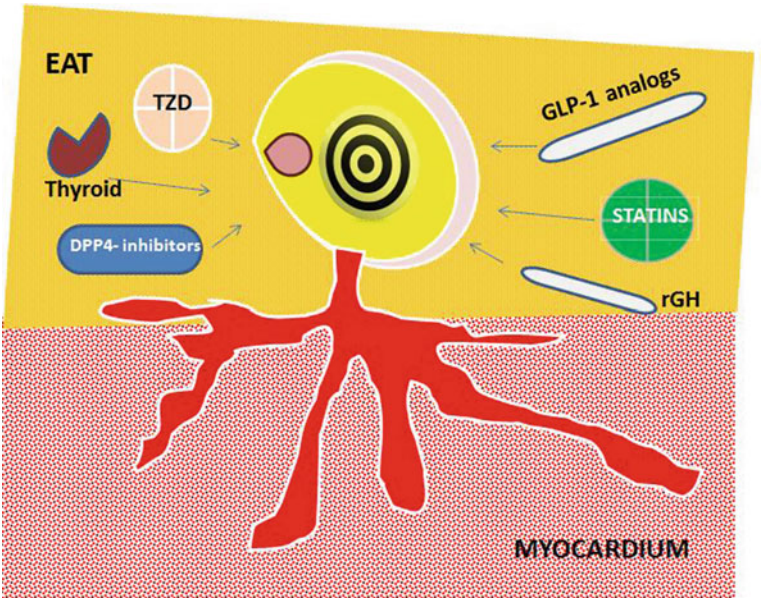

Fig. 6 Epicardial fat as target of drugs modulating the fat. Given its rapid metabolism and rapid changes, epicardial adipose tissue (EAT) is a therapeutic target of medications targeting the fat, such statins, thiazolidinediones (TZDs), dipeptidyl peptidase-4 (DPP4) inhibitors, recombinant growth hormone (rGH), and potentially of glucagon like peptide-1 (GLP-1) analogs and thyroid hormones. These drugs can modulate EAT, reduce its thickness or volume and restore its physiological role

(Alexopoulos et al. 2013; Kim et al. 2009; Park et al. 2010) (Fig. 6). Future studies in this direction are warranted.

Potential Applications

Epicardial fat is an emerging biomarker of visceral adiposity and cardiovascular risk. Its simple and readily available measurement provides both clinician and researcher with a new diagnostic tool. The potential of modulating the epicardial fat to its physiological functions with targeted pharmacological agents can open new avenues in the pharmacotherapy of cardiometabolic diseases.

Summary Points

- Epicardial fat plays a role in the development and progression of cardiometabolic diseases.
- Epicardial fat and its secretosome display local and systemic effects.
- Epicardial fat can be easily measured with imaging procedures, such as echocardiography, magnetic resonance, and computed tomography.

- Epicardial fat assessment is an additional tool for the cardiovascular risk stratification and prediction.
- Epicardial fat measurement can serve as therapeutic target during weight loss and pharmacological interventions targeting the adipose tissue.

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Electrocardiographic Markers of Torsadogenicity

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_8

Abstract

Torsades de pointes is a potentially lethal ventricular tachycardia which has been associated with QT interval prolongation. Although several sophisticated methods have been used in the assessment of proarrhythmic risk, the standard 12-lead surface electrocardiogram still represents the most common and familiar clinical tool. The risk of arrhythmias in several pathological conditions, as well as the torsadogenic potential of drugs, can be assessed noninvasively by the use of electrocardiographic markers. Even though the QT interval duration is the most well known, a few other more accurate markers, such as JT duration, QT dispersion and variability, Tpeak-to-Tend duration, T wave alternans, TRIaD, and beat-to-beat variability of repolarization, have gradually been introduced into clinical practice and have partly replaced or complemented the classic QT interval duration approach. All these electrocardiographic markers are mainly used for noninvasive risk stratification of torsades de pointes and sudden cardiac death in patients with arrhythmogenic syndromes, such as long QT and Brugada syndrome, and also for assessment of new drugs' arrhythmogenic potency.

Keywords

Torsades de pointes • Arrhythmogenicity • Electrocardiographic biomarkers • QT interval • JT interval • Tp-e interval • QT dispersion • Variability of repolarization • T wave alternans • TRIaD

Abbreviations

AP	Action potential
APD	Action potential duration
Bpm	Beats per minute
BVR	Beat-to-beat variability of repolarization
CPVT	Catecholaminergic polymorphic ventricular tachycardia
ECG	Electrocardiogram
HR	Heart rate
ICD	Implantable cardioverter defibrillator
IKr	Rapid component of delayed rectifier K^+ current
LQTS	Long QT syndrome
MTWA	Microvolt T wave alternans
QTd	QT dispersion
QTVI	QT variability index
SQTS	Short QT syndrome
STEMI	Myocardial infarction with ST elevation
STV	Short-term variability
TdP	Torsades de pointes
TDR	Transmural dispersion of repolarization
Tp-e	Tpeak-to-Tend interval
TRIaD	Triangulation, reverse use dependence, instability, and dispersion of repolarization
TWA	T wave alternans

Key Facts of Repolarization Prolongation and Torsadogenicity

- In the myocardial cell, depolarization and maintenance of plateau phase are primarily mediated by Na^+ and Ca^{++} inward currents, while repolarization mainly involves an outward K^+ current.
- The main outward current of repolarization is the delayed rectifier K^+ current (IK) with a slow (IKs) and a rapid (IKr) component.
- Genetic or acquired defects or drugs associated with inhibition of IK current, mostly IKr component, can cause prolongation of phase 3 of the action potential (repolarization).
- Prolongation of repolarization manifests on the surface ECG as QT or JT prolongation and possibly distortion of T waves and presence of prominent U waves.
- When repolarization is prolonged, activation of inward depolarizing currents may generate early after depolarizations, which in turn can induce ventricular extrasystoles if they reach the required voltage threshold.
- In myocardial areas with inhomogeneous cell refractoriness, the mechanism of reentry is facilitated, and torsades de pointes triggered by R-on-T extrasystoles can be maintained via reentrant circuits.

Definitions

Brugada syndrome It is an inherited cardiac channelopathy which follows an autosomal dominant mode of transmission. Patients with Brugada syndrome are at risk of sudden cardiac death due to polymorphic ventricular tachycardia and ventricular fibrillation.

Catecholaminergic polymorphic ventricular tachycardia (CPVT) It is an inherited cardiac disease following an autosomal dominant mode of transmission. It is associated with adrenergic-dependent ventricular tachyarrhythmias. Patients with CPTV are at risk of ventricular tachycardia, ventricular fibrillation, and sudden cardiac death.

Long QT syndrome It is a congenital or acquired arrhythmogenic disorder characterized by dysfunction of cardiac ion channels and prolongation of QT interval on the ECG. The congenital form is inherited via an autosomal dominant or recessive pattern. The syndrome is characterized by a high risk of torsades de pointes, ventricular fibrillation, and sudden cardiac death.

New York Heart Association (NYHA) classification A functional classification for the patients with heart disease. It includes four categories according to the severity of heart failure symptoms and consequent limitation in ordinary physical activity: Class I no symptoms or activity limitation, Class II mild symptoms/activity limitation, Class III marked symptoms/activity limitation, Class IV symptoms even at rest/severe activity limitation.

Short QT syndrome It is an inherited arrhythmogenic disease following an autosomal dominant mode of transmission. It is characterized by a short QT interval on the ECG, atrial or ventricular arrhythmias – such as ventricular tachycardia and fibrillation – and sudden cardiac death.

Introduction

Torsades de pointes (TdP) is a potentially lethal ventricular tachycardia which has been traditionally associated with QT interval prolongation (Antzelevitch and Burashnikov 2001). It may become self-terminated within seconds or progress to ventricular fibrillation, thus leading to sudden cardiac death (Gupta et al. 2007). The term "torsades de pointes" is French and literally means "twisting of the points" or "twisting of the spikes"; it characteristically describes the electrocardiographic (ECG) sinusoidal pattern of this arrhythmia with polymorphic QRS complexes twisting around the isoelectric baseline (Fig. 1).

Two electrophysiological mechanisms are considered to be involved in the development of TdP: early after-depolarization triggered activity which results in the genesis of a premature beat and amplification of transmural dispersion of repolarization which creates the window for reentry (Antzelevitch and Burashnikov 2001). Factors that enhance the intrinsic electrical heterogeneity within ventricular myocardium may trigger the development of TdP (Antzelevitch 2005; Antzelevitch and Burashnikov 2001). The presence of electrical heterogeneity under baseline conditions is associated with the different properties of three cell types in the ventricular myocardial wall: epicardial, endocardial, and midmyocardial cells (M cells), (Issa et al. 2012). Epicardial cells have the shortest, while M cells have the longest action potential duration (APD) (Antzelevitch 2005; Gupta et al. 2007; Issa et al. 2012). Furthermore, in the presence of APD-prolonging factors, such as bradycardia or specific drugs, the M cells are more susceptible to further prolongation of their APD than the other cells (Issa et al. 2012). In myocardial areas where cells have significantly different APD, thus different refractoriness, early after depolarizations may induce R-on-T extrasystoles that trigger initiation of TdP which is maintained via reentrant circuits (Antzelevitch 2005; Gupta et al. 2007; Issa et al. 2012).

Even though several sophisticated markers and invasive tests have been used as predictors of TdP, the 12-lead ECG still represents the most common and familiar tool advocated in clinical practice (Gupta et al. 2007). Each ECG wave recorded on body surface represents an electrical voltage gradient generated in the heart; the QRS complex manifests ventricular depolarization, while the ST segment and J, T, and U waves reflect ventricular repolarization (Yan et al. 2003).

Among ECG torsadogenic markers, the QT interval prolongation is the most well known. Nevertheless, it actually represents only a surrogate index, associated with a different risk of TdP in various conditions and patient groups (Staikou et al. 2014). On the other hand, a few other ECG parameters, such as JT duration,

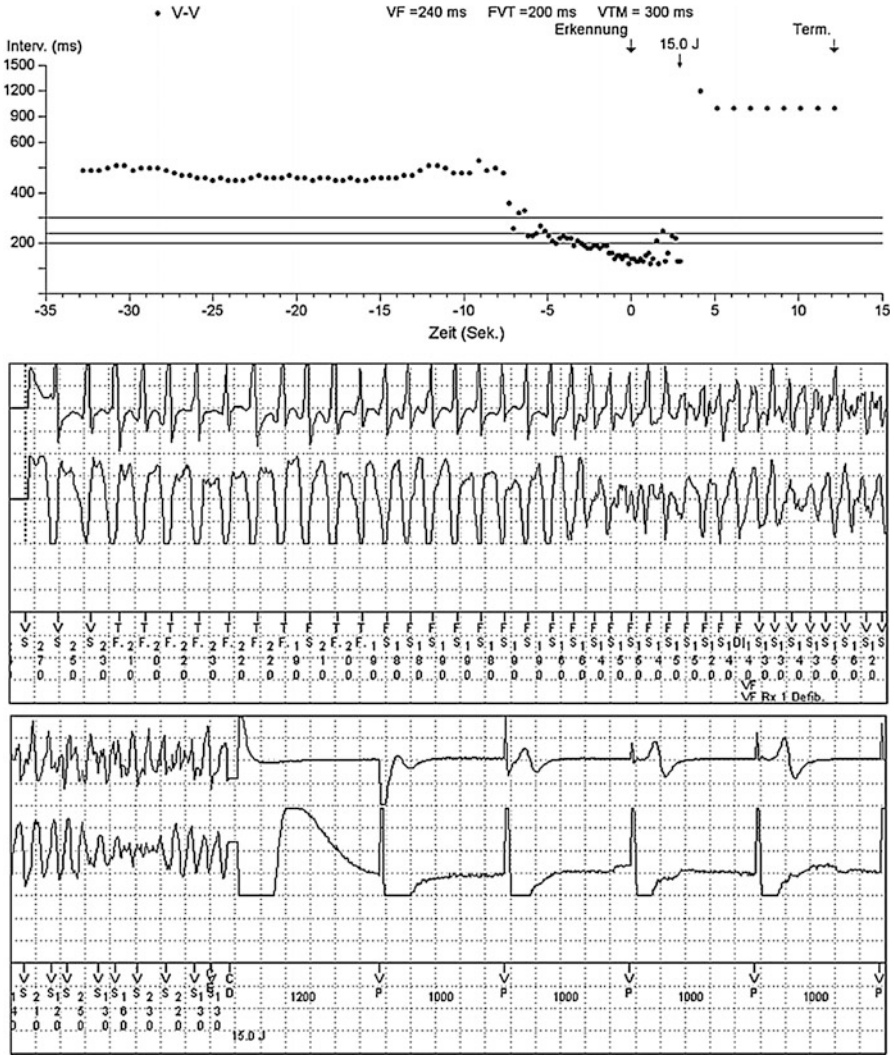

Fig. 1 Torsades de pointes ventricular tachycardia successfully defibrillated. The electrocardiogram of a patient who developed torsades de pointes ventricular tachycardia with the characteristic sinusoidal pattern. The arrhythmia was successfully terminated before degenerating into ventricular fibrillation by discharge of patient's implantable cardioverter defibrillator at 15 J (Figure from Krause et al. 2011; With kind permission from Springer Science+Business Media: Clin Res Cardiol., A rare association of long QT syndrome and syndactyly: Timothy syndrome (LQT 8), Vol. 100, 2011, p. 1123–1127, Krause, Gravenhorst, Kriebel et al., Fig. 4)

QT dispersion and variability, Tp-e duration, T wave alternans, TRIaD, and beat-to-beat variability of repolarization, are considered superior and have partly replaced or complemented the classic QT interval duration approach (Staikou et al. 2014).

Torsadogenic Markers: Description and Measurement

QT Interval

The duration of QT interval on the surface 12-lead ECG has long been used as a marker of torsadogenicity. The interval is measured from the beginning of QRS complex to the end of T wave, thus corresponding to the phases of ventricular depolarization and repolarization. The QT duration is mainly determined by M-cell repolarization, since these cells have the longest APD and the completion of their repolarization coincides with the end of T wave (Antzelevitch 2008). Any APD prolongation (either due to depolarization or more often due to repolarization lengthening) manifests as QT interval prolongation (Issa et al. 2012).

On the surface 12-lead ECG, the QT interval is preferably measured in lead II and precordial leads (Garson 1993; Gupta et al. 2007; Moss 1993; Staikou et al. 2014). The presence of U waves may interfere with the measurement of QT interval. On the ECG, the U wave reflects the last phase of ventricular repolarization and is seen after T wave as a physiologic small deflection or a more distinct pathological wave (Yan et al. 2003). When a discrete, separate U wave follows the T wave, it should not be calculated as part of QT interval (Moss 1993). When pathological large U waves merge with the T wave, then the QT interval measurement should include them and not only the first component of the T wave (Yan et al. 2003). In order to overcome the measurement difficulties due to U waves, lead II is preferred because the U waves are less prominent there and usually a long single wave is observed (Garson 1993).

Different methods have been proposed for the determination of T wave end (Fig. 2). The visual method is the simplest and defines the end of T wave as the point where the descending limb returns to the isoelectric baseline. Another popular method is the tangent method, which determines the T wave end as the point where the tangent through the steepest downslope of the wave intersects the isoelectric line (Isbister and Page 2013). The tangent method is characterized by a relatively low inter-reader variability, but it may under-measure the interval and is less accurate in the presence of T waves with morphological abnormalities (Isbister and Page 2013). Manual determination of T wave end and consequently of QT interval duration is more accurate than automatic measurements which are provided by the standard 12-lead ECG machines (Fig. 3). Thus, in suspicious cases, it is suggested that the QT interval should be preferably measured manually, as described in Table 1 (Gupta et al. 2007; Isbister and Page 2013).

The duration of QT interval is influenced by the heart rate (HR): bradycardia prolongs it, while tachycardia shortens it. In order to minimize the HR dependence, the QTc which is rate corrected is usually preferred. For the calculation of QTc, several different formulas have been proposed; among the most popular ones are those of Bazett $[(QT/(RR)^{1/2}]$ and Fridericia $[(QT/(RR)^{1/3}]$ which are logarithmic (log linear) and those of Framingham $[QT + 0.154\,(1 - RR)]$ and Hodges $[QT + 1.75\,(HR - 60)]$ which are linear (Staikou et al. 2014). In the above formulas,

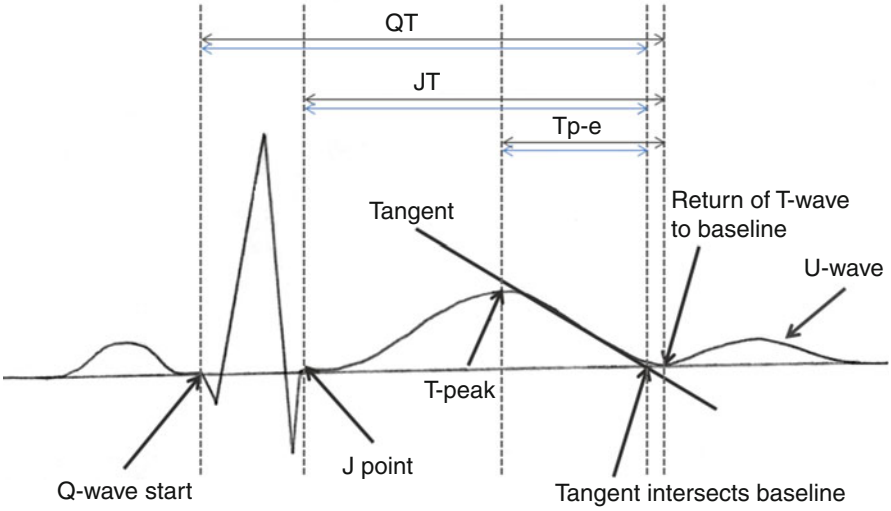

Fig. 2 Identification of specific points on the electrocardiogram for manual measurement of QT, JT, and Tp-e intervals. The points that should be identified on the electrocardiogram for manual measurement of the intervals are Q wave start, J point, Tpeak, and Tend. Regarding the T wave end, according to the visual method, it is the point where the descending limb of the wave returns to the isoelectric line, while according to the tangent method, it is the point where the tangent through the steepest downslope of the wave intersects the isoelectric line

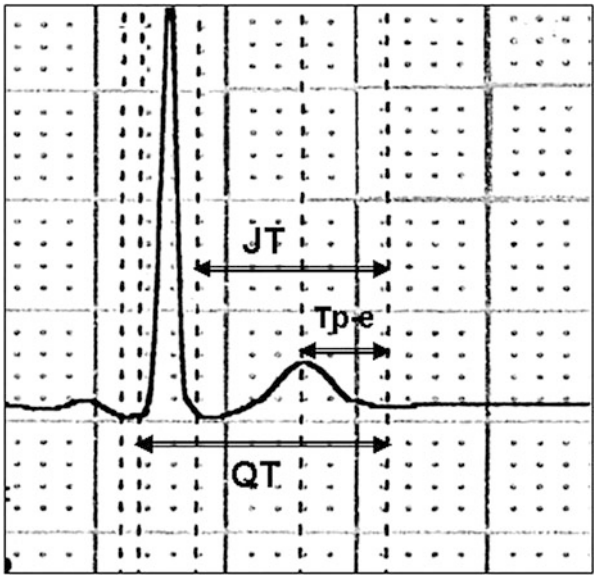

Fig. 3 Automatic measurement of QT, JT, and Tp-e intervals by the electrocardiographic machine. The QT, JT, and Tp-e intervals measured on a lead of the standard electrocardiogram. The *vertical black dotted lines* are marked on the paper by the electrocardiograph, and the interval measurements are provided automatically by the machine

Table 1 Method for manual measurement of QT interval. Guiding steps for correct manual measurement of QT interval on the standard 12-lead surface electrocardiogram

Measurement of QT interval (preferably by the visual method)	From beginning of Q wave to the end of T wave (return to the isoelectric line)
Assessment of QT interval in six ECG leads	In three limb leads: preferably I, II, and aVF or aVL
	In three precordial leads: preferably in V2, V4, V6 (V3 and V5 may be also used alternatively)
	The measurements should be averaged over 3–5 beats in a single lead
	The median of the six leads is calculated
Leads to avoid due to difficulty in QT assessment	V1
	Lead III: usually of low voltage
	aVR: inverted
QT measurement in the presence of U waves	If prominent U waves merge into the preceding T wave, they should be included in the QT measurement
QTc evaluation	The measurements should be corrected for the HR

the RR interval equals to 60/HR, because the QTc estimates the QT interval at 60 beats per minute (bpm). The Bazett's formula, which is the most simple and well known, undercorrects the QT at HRs below 60 bpm and overcorrects it at HRs above 100 bpm. The linear formulas of Framingham and Hodges are less HR dependent and probably more accurate for a wider range of HRs (Staikou et al. 2014). Still, no formula can completely eliminate the dependence of QT duration on the HR (Isbister and Page 2013).

Regarding normal values, the threshold of 440 ms is generally considered the upper normal limit of QTc. However, population-based studies have shown significant differences between the genders, with women having higher values by about 20 ms (Isbister and Page 2013; Moss 1993; Staikou et al. 2014). Thus, QTc may range from 350 to 450 ms in healthy men and from 360 to 470 ms in healthy women (Moss 1993; Staikou et al. 2014). There are also other factors contributing to the variability observed in QT duration; it exhibits a diurnal variation and is influenced by the wakefulness state, autonomic tone, and age (Garson 1993; Isbister and Page 2013). Higher values are found in people of older age and in sleeping rather than awake individuals with the same HR (Garson 1993), as shown in Tables 2 and 3.

J Wave and JT Interval

The J wave, also known as Osborn wave, is a deflection observed after the QRS complex on the surface ECG. It is usually "buried" in the QRS when the activation spreads from the epicardium to the endocardium, while it is clearly seen when the activation begins in the endocardium and spreads transmurally to the epicardium (Hlaing et al. 2005). On the normal ECG, the JT interval is measured from the point

Table 2 Normal and pathological values of the most common electrocardiographic torsadogenic markers. The reported normal and pathological values of QTc, JTc, QTd, QTVI, Tp-e, and Tp-e/QT

Electrocardiographic marker	Normal values	Pathological values	Values related to arrhythmogenicity
QTc interval	≤440 ms	>470 ms (males)	>500 ms
	350–450 ms (males)	>490 ms (females)	
	360–470 ms (females)	Or change >20 ms	
JTc interval	320 ± 20 ms	>340 ms	Not defined
	Upper limit: 340 ms		
QT dispersion	20–50 ms	>50 ms	>100 ms (to overcome measurement inaccuracies)
QT variability index	Typical normal values <−1	>−0.9	≥0.1
	Reported range: −0.97 to −2.23		
Tp-e interval	40–110 ms	>110 ms	>117 ms
	Mean value: ~76 ms		
Tp-e/QT ratio	0.15–0.25	>0.25	>0.28
	Mean value: 0.21		

QTc QT interval corrected for heart rate, *JTc* JT interval corrected for heart rate, *QTd* QT dispersion, *QTVI* QT variability index, *Tp-e* Tpeak-to-Tend interval

where the QRS complex joins the ST segment (J point) to the end of T wave (Figs. 1 and 2) and corresponds to ventricular repolarization time (Vecht et al. 2009).

The concept of JT interval duration as an arrhythmogenic marker was proposed in order to overcome possible inaccuracies of QT interval approach in certain conditions, since the latter actually reflects the duration of both depolarization and repolarization (Q wave start to T wave end). Physiologically, when ventricular conduction is normal, the QRS duration (usually between 70 and 100 ms) does not affect significantly the duration of QT interval (Spodick 1992). Nevertheless, in cases with prolonged depolarization time (QRS >120 ms), the QT interval will also be significantly prolonged (Salik and Muskin 2013). This methodological problem is mainly encountered in the presence of ventricular conduction defects, where the widening of the QRS complex may prolong the QT interval by up to 16 % (Pickham and Hasanien 2013; Salik and Muskin 2013). Similarly, when depolarization time is too short (very narrow QRS complexes), the QT interval prolongation induced by medications, electrolyte, or metabolic abnormalities will become evident on the surface ECG quite late, only after a significant change has already occurred (Spodick 1992). In such cases, the subtraction of depolarization time (QRS duration) from QT interval measurement will provide a more precise measurement of ventricular repolarization. The JT interval (JT = QT − QRS) is

Table 3 Factors and conditions affecting the values of electrocardiographic arrhythmogenic markers. Factors contributing to the variability of electrocardiographic markers of arrhythmogenicity and pathological conditions which may be characterized by abnormal values, risk of arrhythmias, and sudden cardiac death

Electrocardiographic marker	Factors affecting marker's measured values	Pathological conditions with abnormal values
QT interval	HR	Congenital LQTS
	Gender	Acquired LQTS (cardiac, electrolyte, endocrine abnormalities)
	Age	SQTS
	Autonomic tone	
	Wakefulness state	
	Circadian variation	
	Method of measurement	
JT interval	HR	Congenital LQTS
	Gender	Acquired LQTS
	Method of measurement	SQTS
QT dispersion	Inaccuracies due to methodological issues	Congenital LQTS not responding to beta-blockers
		Myocardial ischemia/infarction
		Hypertension/congestive heart failure/ diabetes complicated with arrhythmias
QT variability index	Age	Congenital LQTS
	Autonomic tone	Acquired LQTS
	Circadian variation	Acute myocardial ischemia
	Method of measurement	Coronary artery disease
		Patients with ICDs
		Impaired cardiac function
		Cardiomyopathy dilated/hypertrophic
		Kawasaki disease
		Familial dysautonomia
		Type 1 myotonic dystrophy
		Spinal cord lesions
		Beta-thalassemia
		Renal disease
		Psychiatric disorders
Tp-to-Tend interval	HR	Congenital LQTS
	Method/lead of measurement	Acquired LQTS
		Brugada syndrome
		SQTS
		Hypertrophic cardiomyopathy
		CPVT
		Acute STEMI
		Acquired bradyarrhythmias

(*continued*)

Table 3 (continued)

Electrocardiographic marker	Factors affecting marker's measured values	Pathological conditions with abnormal values
T wave alternans	HR	Myocardial ischemia
	Autonomic tone	Heart failure
		Hypertrophic cardiomyopathy
Beat-to-beat variability of repolarization	Method of assessment	Congenital LQTS
		Nonischemic congestive heart failure
		Cardiomyopathy
		Patients with ICDs

LQTS long QT syndrome, *SQTS* short QT syndrome, *CPVT* catecholaminergic polymorphic ventricular tachycardia, *STEMI* myocardial infarction with ST elevation, *ICD* implantable cardioverter defibrillator

relatively unaffected by changes in QRS duration and thus represents a more specific marker of repolarization abnormalities compared with QT interval duration (Salik and Muskin 2013). Since JT values may be influenced by the HR, the corrected interval (JTc) defined as the Bazett's [QTc – QRS] is preferred as a more accurate index (Staikou et al. 2014).

Although clear normal limits of JT interval duration have not been defined (Pickham and Hasanien 2013), the reported mean JTc values in healthy population are 320 ± 20 ms, and measurements up to 340 ms are associated with normal repolarization times (Vecht et al. 2009), (Table 2). Differences have been found among genders: healthy men have shorter JT intervals than women, probably because of the effects of testosterone on repolarization (Sgarbossa and Wagner 2007). Regarding pathological values, a JT index formula has been proposed $[\text{JT index} = \text{JT}\,(\text{HR} + 100)/518]]$, with values ≥112 ms indicating prolongation of repolarization (Pickham and Hasanien 2013). In any case, the arrhythmogenic potential of an intervention should better be assessed via serial measurements of JT interval and comparison with baseline values (Pickham and Hasanien 2013).

QT Dispersion

The QT dispersion (QTd) expresses the inter-lead QT variation and represents another possible noninvasive indicator of proarrhythmic risk. It is defined as the difference between the maximum and minimum values of QT interval duration (QTd = QTmax−QTmin) as measured on a 12-lead standard ECG and reflects the dispersion of recovery of ventricular excitability due to myocardial inhomogeneity. Despite a great interindividual variability (10–71 ms), the marker does not seem to be significantly affected by age or gender. In a healthy population, QTd measurements are usually in the range of 20–50 ms, and values exceeding the threshold of 50 ms are considered abnormal (Higham and Campbell 1994; Malik and Batchvarov 2000), (Table 2).

Unlike QT interval, the dependence of QTd on HR has never been adequately established, and thus, the index is not usually rate corrected (Malik and Batchvarov 2000). However, the HR corrected QTd (QTcd) has been used as an indicator of torsadogenicity in experimental studies (Bluzaite et al. 2006; Staikou et al. 2014).

Methodological difficulties, such as errors in QT interval estimation due to unclear recording of QRS start and T wave end or weak projection of complicated three-dimensional T wave loop in individual ECG leads, can significantly affect the accuracy of QTd (Shah 2005; Surawicz 1996). Furthermore, the introduction of other markers, such as Tp-e, has further limited the use of QTd as a tool of assessing the intramyocardial dispersion of repolarization (Shah 2005).

QT Variability Index

Another concept which turned up to be more accurate than QT interval duration in predicting the risk of TdP is the assessment of QT interval variability, which may be short or long term. The QT variability index (QTVI) is one of the nine QT interval variability markers which are categorized in four groups according to the consecutiveness or not of the measured beats and normalization or not of HR variability (Niemeijer et al. 2014).

The QTVI combines the fluctuations of QT interval and HR in the following equation: $\mathrm{QTVI} = \mathrm{Log}_{10}\left[\left(\mathrm{QTV}/\mathrm{meanQT}^2\right)/\left(\mathrm{HRV}/\mathrm{meanHR}^2\right)\right]$, where QTV is the normalized QT variance and HRV is the normalized HR variance (Berger 2003). This log ratio of the two normalized variables has no measurement units, and its typical normal values are below -1 (Dobson et al. 2013). In a healthy population, QTVI values have been found in the range of -0.97 to -2.23 (Dobson et al. 2013) (Table 2). The index increases with age and follows a circadian pattern with lowest values at night and peak values around noon (Dobson et al. 2013).

The QTVI seems to provide useful information about the beat-to-beat repolarization lability (Berger 2003). More precise results can be drawn after 256 s of ECG recording without the need for tachycardia (Thomsen et al. 2006); thus, the index is advantageous as the measurements are not necessarily performed under stressing conditions or exercise (Dobson et al. 2013). Nevertheless, the sensitivity to probable erroneous QT measurements is relatively low (Tereshchenko and Berger 2011).

Elevation of QTVI values may be due to a rise in QT variance or a drop in HR variance (Berger 2009). There is also a direct relation of the index with the sympathetic tone, but only under pathologically stressful conditions, such as congestive heart failure (Berger 2009). Increased values indicate amplification of temporal repolarization lability and are associated with a high risk of malignant ventricular arrhythmias, such as ventricular tachycardia or fibrillation, and sudden cardiac death (Staikou et al. 2014). However, a specific cutoff point associated with arrhythmogenicity has not been defined due to methodological difficulties.

Transmural Dispersion of Repolarization, Tp-e Interval, and Tp-e/QT Ratio

The T wave reflects ventricular repolarization on surface ECG; its peak point coincides with completion of epicardial repolarization, and its end point with full recovery of M cells (Antzelevitch 2001; Gupta et al. 2008). Thus, the APD of epicardial cells determines the Q-to-Tpeak interval duration, while the APD of M cells determines the Q-to-Tend interval duration (Antzelevitch 2001). The Tpeak-to-Tend interval (Tp-e) is a significant ECG torsadogenic marker, as it may reflect the transmural dispersion of repolarization (TDR) (Antzelevitch 2001). In the presence of upright T waves, it is measured from the point where the deflection of the wave reaches its highest amplitude (T wave peak), while in the presence of a more complicated configuration, such as negative or biphasic T waves, the Tp-e interval is measured from the lowest point of the first component to the final end of the wave (Antzelevitch 2001; Antzelevitch 2007; Gupta et al. 2008).

Even though the Tp-e interval duration is considered to correlate with TDR, it has not been directly validated as an accurate, specific TDR measure (Antzelevitch 2007; Gupta et al. 2008). It has been suggested that Tp-e may probably reflect better the total dispersion of ventricular repolarization (transmural, apicobasal, global) than TDR alone (Antzelevitch 2007; Gupta et al. 2008). Increased TDR values are associated with a steep repolarization gradient which in turn is related to arrhythmogenicity. On the other hand, apicobasal and interventricular dispersion of repolarization are not necessarily associated with a steep gradient and arrhythmogenic potency (Antzelevitch 2007). For the aforementioned reasons, it is suggested that the Tp-e interval should be preferably measured in the precordial leads, which represent more accurately the TDR compared to limb leads (Antzelevitch 2007). The precordial unipolar leads are in close proximity to the heart and record the electrical activity in the horizontal plane, looking across the right (V1, V2) and left (V5, V6) ventricular wall (Antzelevitch 2007). Thus, the lead V6 may reflect better the transmular axis of the left ventricle (Gupta et al. 2008). Additionally, since TDR may differ significantly among various myocardial areas, the Tp-e interval should be measured in each precordial lead for a more accurate and valid assessment. It is also suggested that the measurements in a number of different leads should not be averaged (Antzelevitch 2008). According to the existing cardiac pathology, TDR increases may be found either in left or in right precordial leads; for example, in LQTS (left ventricle pathology), it would be more appropriate to assess the Tp-e in left precordial leads (i.e. V5), while in Brugada syndrome (right ventricle pathology), the interval should preferably be assessed in right precordial leads (i.e. V2), (Antzelevitch 2007).

In a healthy population with a HR between 60 and 100 bpm, Tp-e values range between 40 and 110 ms (mean value of 76.1 ms) (Gupta et al. 2008). The marker exhibits significant interindividual variability and is HR dependent according to an inverse linear relationship. The Tp-e/QT ratio is another arrhythmogenic index superior to Tp-e interval duration, because it is less influenced by the HR; for the

aforementioned range of HRs, the Tp-e/QT values remain relatively constant between 0.15 and 0.25 (mean value of 0.21) (Gupta et al. 2008) (Table 2). An increase of Tp-e/QT ratio, showing that Tp-e interval is more prolonged than QT interval, is critical for the development of ventricular arrhythmias (Gupta et al. 2008). Increases in Tp-e and Tp-e/QT values correspond to amplification of dispersion of repolarization (TDR or global) which creates a vulnerable window for early after-depolarization induced extrasystoles, thus increasing the risk for ventricular arrhythmias (Antzelevitch 2005; Staikou et al. 2014).

T Wave Alternans

Morphological variations in ECG components were initially observed with the naked eye and mainly involved the phase of repolarization, especially T wave. Since 2006, T wave alternans (TWA) has been adopted as a class IIa marker for stratification of malignant arrhythmias according to the guidelines of the American College of Cardiology, American Heart Association, and European Society of Cardiology (Zhang et al. 2011). The term microvolt T wave alternans (MTWA) stands for the beat-to-beat variations in ST segment or T wave morphology and amplitude (Nieminen and Verrier 2010). It is the result of repolarization derangements in APD and reflects the spatial or temporal repolarization inhomogeneity (Cutler and Rosenbaum 2009). At microscopic level, it has been found that even minimum values of these electrocardiographic alternans may represent important cellular repolarization modifications and potential risk of sudden cardiac death (Cutler and Rosenbaum 2009). According to the electrophysiological defects, ECG fluctuations may involve different parts of T wave, such as the initial half of T wave in subendocardial ischemia or variations above and below the isoelectric line in LQTS (Nieminen and Verrier 2010).

Identification of TWA is made by the use of computerized methods, with the spectral and modified moving average being the most important. The former requires specially designed ECG electrodes for the recordings and uses a fast Fourier transform method to analyze the results of 128 adjacent beats at a HR frequency of 105–110 bpm, thus simulating stress conditions. In the second method, specific electrodes or a fixed high HR are not required, and the measurements can be made under normal conditions during routine evaluation, in ambulatory patients, or in symptom-limited exercise stress testing (Floré and Willems 2012; Verrier and Malik 2013). The existing data suggest that the predictive value of the two methods is similar, even though the spectral method has been applied more extensively (Nieminen and Verrier 2010; Verrier et al. 2011).

Two potential mechanisms have been proposed to induce repolarization variations and explain cardiac myocyte alternans; according to APD restitution hypothesis, voltage or AP morphologic changes can influence intracellular Ca^{++} levels, while Ca^{++} restitution hypothesis suggests fluctuating Ca^{++} transients as the initial event before the occurrence of sarcolemmal, voltage, and AP morphologic alternations (Merchant et al. 2013). In fact, none of the above mechanisms alone can

explain the ECG alternans, while it is more likely that they are both implicated in the underlying pathophysiology (Floré and Willems 2012).

TRIaD

A number of repolarization derangements incorporated into the concept of TRIaD (triangulation, reverse use dependence, instability, and dispersion of repolarization) have attracted the interest of investigators as reliable tools in proarrhythmic risk assessment, especially when combined with classic ECG torsadogenic markers, such as QT interval prolongation.

The term "triangulation" refers to the shape of monophasic AP due to prolongation from 30 % to 90 % completion of repolarization (time from APD_{30} to APD_{90}). The triangular shape of AP contour results from a decrease or blockade of outward repolarizing currents or from augmented inward depolarizing currents. In any case, triangulation is supposed to be proarrhythmic as it represents a slower repolarization during which hibernated Ca^{++} and Na^{+} currents may become reactivated and depolarize the myocardium (Thomsen et al. 2006). The second component of TRIaD named "reverse use dependence" refers to APD prolongation due to class III antiarrhythmic drugs which is attenuated as HR rises. Finally, beat-to-beat alterations in APD known as instability and spatial or temporal dispersion of refractoriness represent the last two elements in TRIaD theory (Hondeghem 2008; Shah and Hondeghem 2005; Thomsen et al. 2006).

The ECG counterparts of triangulation (T) are wider, smoother, and notched T waves, while reverse use dependence (R) appears as a steep QT-RR slope; characteristically, amiodarone – which is a relatively safe class III antiarrhythmic drug – has minimum effects on QT-RR slope. Instability of APD (I) manifests on the ECG as labile QT interval or TWA with or without rhythm abnormalities that come first and augment the whole beat-to-beat ADP variation. Last but not least are dispersion (D) indices due to inhomogeneity of refractoriness throughout the heart (Hondeghem 2006; Shah and Hondeghem 2005). The presence of the above ECG components of TRIaD is associated with increased proarrhythmic risk, whereas their absence yields reduced TRIaD and antiarrhythmia.

Beat-to-Beat Variability of Repolarization Duration

The beat-to-beat variability of repolarization (BVR) is another potential index of arrhythmogenicity arising from recent experimental trials. It is quantified as short-term variability (STV) and is a measure of temporal variability of repolarization duration among a number of consecutive heartbeats (Varkevisser et al. 2012). The underlying pathophysiology involves random potassium currents (I_K) which are suspected to play an important role, especially when repolarization reserve is diminished, as in case of acidosis, ischemia, or pharmacological I_{Kr} inhibition (Floré and Willems 2012; Oosterhoff et al. 2007; Thomsen et al. 2006).

For BVR assessment, the QT intervals or APDs of a number of consecutive beats (usually 30 beats) are plotted against their duration in the preceding beats in a Poincaré plot (Thomsen et al. 2006; Varkevisser et al. 2012. The BVRs from different patients or new chemical entities can be efficiently compared when quantified as STV, which represents the width of the Poincaré plot, i.e., the mean dispersion of repolarization time of the consecutive beats from the line of identity. The STV can be derived from the following formula, $\left[\mathrm{STV} = \sum |D_{n+1} - D_n| / \left(N \times \sqrt{2}\right)\right]$, where D is the duration of repolarization and N the number of consecutive beats and is expressed in units of time (ms) (Varkevisser et al. 2012. Credible measurement of STV requires ECG recording of at least 2 min, and new software should be applicable for the semiautomatic analysis of the results.

Torsadogenic Markers: Potential Applications to Prognosis, Other Diseases, or Conditions

QT Interval

The QT interval duration has been used for decades as an index of arrhythmogenicity in various pathological conditions (Table 3). QTc values above 450 or 470 ms in men and women, respectively, are considered increased (Isbister and Page 2013), and a prolongation over the limits of 470/490 ms in men/women or a change of more than 20 ms from baseline after an intervention increases the risk for TdP and sudden cardiac death (Bednar et al. 2001; Staikou et al. 2014). A QTc interval exceeding the threshold of 500 ms is considered a major risk factor of TdP (Bednar et al. 2001; Isbister and Page 2013) (Table 2).

The QT interval may be found pathologically prolonged in congenital or acquired conditions characterized as long QT syndromes (LQTS) (Fig. 4). In patients with LQTS, preferential prolongation of M-cell APD results in QT interval prolongation, notched T waves, and pathologic U waves on the ECG (Antzelevitch 2007, 2008). The above mechanism also explains the torsadogenic susceptibility of these patients (Yan et al. 2003). In congenital types of LQTS, there are several genotypes associated with cardiac channel dysfunctions and different clinical presentations. In these cases, the ECG may be helpful in diagnosis and subtype determination; a prolonged or borderline QTc interval should be investigated, especially in the presence of suspicious symptoms and sudden cardiac death in the family (Staikou et al. 2012). In acquired forms of LQTS, the possible causative factor, such as electrolyte abnormality, cardiac disease, endocrine disorder, and drugs, should be investigated and corrected if possible (Staikou et al. 2014). Specifically, hypokalemia, hypocalcemia, hypomagnesemia, hypertension, myocardial ischemia or failure, cardiomyopathies, bradycardia due to sinus node dysfunction or conduction abnormalities, Kawasaki syndrome, thyroid disorders, diabetes mellitus, and obesity have all been associated with QT prolongation (Isbister and Page 2013; Staikou et al. 2014).

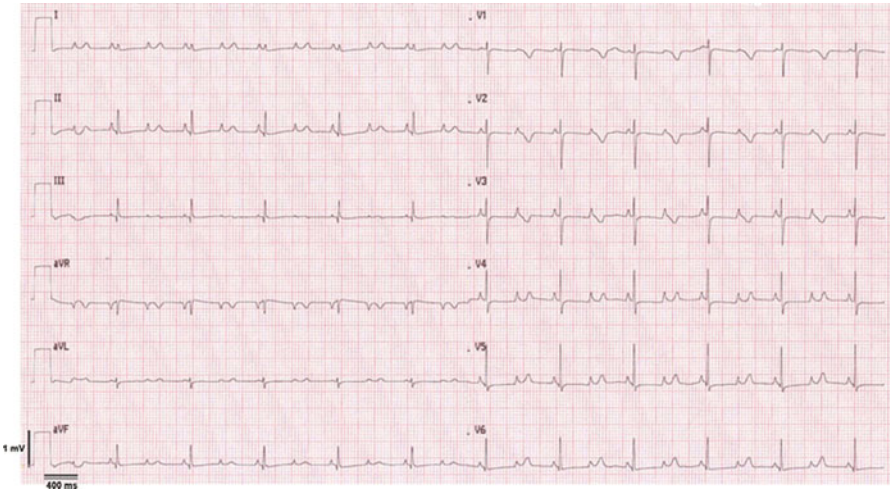

Fig. 4 Significant QT interval prolongation identified on a standard 12-lead surface electrocardiogram. The electrocardiogram of a patient with Timothy syndrome (long QT syndrome type 8), characterized by QT prolongation and syndactyly. The recording shows a QTc of 600 ms, 2:1 atrioventricular block, and a ventricular rate of 60 bpm (Figure from Krause et al. 2011; With kind permission from Springer Science+Business Media: Clin Res Cardiol., A rare association of long QT syndrome and syndactyly: Timothy syndrome (LQT 8), Vol. 100, 2011, p. 1123-1127, Krause, Gravenhorst, Kriebel et al., Fig. 2)

The duration of QT interval has also been used as a safety profile marker in the process of approval and drug labelling; certain antiarrhythmics, antipsychotics, antidepressants, antihistamines, antimicrobials, and gastrokinetics have been implicated in QT prolongation (Isbister and Page 2013; Staikou et al. 2014). An updated list of QT-prolonging drugs stratified by relative risk can be found in electronic databases (www.torsades.org, www.qtdrugs.org, www.longqt.org, www.sads.org) (Gupta et al. 2007). The duration of QT interval and its relative changes are assessed for therapeutic or high drug doses and also for drug interactions. The individual risk for each patient can be assessed via manual measurement of QT interval and plotting the QT values against HR on a QT nomogram (Isbister and Page 2013). Also, evaluation of the risk/benefit ratio is performed for QT-prolonging drugs that are known to have significant benefits for some patients, such as methadone. In these cases, a pretreatment ECG or preferably sequential recordings during the day or 24-h Holter recordings are needed as baseline. The follow-up includes ECG recordings while the patient is on drug therapy and QT comparisons with baseline measurements (Isbister and Page 2013).

The assessment of QT interval duration on a standard surface ECG is also significant in cases with suspected short QT syndrome (SQTS). Individuals with a QT interval duration of less than 350 ms should be further investigated, while in those with abnormally short intervals (<320 ms), the possibility of SQTS should be strongly considered (Bjerregaard and Gussak 2008). For the diagnosis of

SQTS, the HR should be less than 130 bpm – ideally below 100 bpm – for improved accuracy in QT interval measurements (Bjerregaard and Gussak 2008). Another index, the QT/RR ratio is helpful in distinguishing the individuals with a short QT who are susceptible to arrhythmias; a ratio of <0.1 may be indicative of a high risk of ventricular fibrillation and sudden cardiac death (Bjerregaard and Gussak 2008).

J Wave and JT Interval

The J wave and JT interval may be useful when assessing patients with life-threatening arrhythmogenic syndromes and ventricular conduction abnormalities. A prominent J wave is often seen in individuals with Brugada syndrome, idiopathic ventricular tachycardia, and early repolarization syndrome (Yan et al. 2003), while high JTc values (>340 ms) are found in patients with LQTS (Table 3). In Brugada syndrome, a common ECG finding is an accentuated J wave which presents as an incomplete right branch bundle block (V1–V3 precordial leads). Notably, a J wave combined with ST elevation in V1–V3 leads (right ventricle) is associated with arrhythmogenicity, as in Brugada syndrome, while a J wave and ST elevation in V4–V6 leads (left ventricular anterolateral wall) are benign and are found in early repolarization syndrome (Hlaing et al. 2005).

In the presence of ventricular conduction defects, especially complete bundle branch bock (QRS duration ≥ 120 ms), the JT interval reflects the duration of ventricular repolarization time more accurately than the QT interval, by eliminating QRS duration (Salik and Muskin 2013). In the presence of incomplete bundle branch block (QRS duration: 100–120 ms), although the influence on QT duration is less significant, JT interval is also preferred as an index due to the difficulties encountered in measuring QT interval (Salik and Muskin 2013). The marker is also useful in patients with excessively narrow QRS complexes, as in obstructive lung disease where QRS duration may be of about 50 ms. In these cases, the QT interval may even increase by up to 40 ms before its prolongation becomes evident on the surface ECG. Consequently, the JT interval measurement helps in early recognition of arrhythmogenicity of various QT-prolonging drugs (Spodick 1992).

QT Dispersion

Despite many controversies, there are sufficient data to support the correlation of QTd with myocardial ischemia and malignant ventricular arrhythmias (Bluzaite et al. 2006) (Table 3). QTd elevations between 60 and 100 ms have been observed after myocardial infarction (Higham and Campbell 1994) with a tendency to decrease after percutaneous transluminal coronary angioplasty (Sredniawa et al. 2001). Even higher values, in the range of 130–200 ms, are found in LQTS, especially in patients with the congenital type who do not respond to beta-blockers

(Antzelevitch et al. 1998). Abnormally high values have been related to increased cardiovascular mortality (Bluzaite et al. 2006) and a high risk of sustained ventricular tachycardia in hypertension, congestive heart failure, and diabetes (Sredniawa et al. 2001). On the other hand, its problematic reproducibility, difficulties in measuring ECG complexes and waves, poor standardization, and low sensitivity and specificity are some of the factors that limit the clinical usefulness if this marker (Antzelevitch et al. 1998; Balaji et al. 2002; Haugaa et al. 2011). Currently, QTd is mainly considered as a simple and indirect index of undefined repolarization alterations and defects (Kittnar and Lechmanová 2001; Malik and Batchvarov 2000). Its clinical utility has been limited to cases with high-enough values – i.e., exceeding 100 ms – to override any possible measurement faults and also for assessing the safety profile of drugs that are known to elongate ventricular repolarization (Malik and Batchvarov 2000).

QT Variability Index

High QTVI values are found not only in cardiac diseases but also in several other abnormal states (Table 3). Both congenital and drug-induced LQTS are characterized by increased QTVI values, and the index can be used for the assessment of certain drugs' arrhythmogenic profile (Thomsen et al. 2006). Values ≥ 0.1 have been associated with a high risk of future malignant arrhythmias in patients undergoing electrophysiological investigation (Staikou et al. 2014). A QTVI of -0.27 is found in patients with ICDs, with values exceeding 0.14 indicating a high risk of sudden death (Dobson et al. 2013).

An index higher than -0.47 seems to be related with mortality in New York Heart Association (NYHA) I patients with mildly affected ventricular contractility (Dobson et al. 2013). Values up to 0.14 have been found in acute myocardial ischemia (Dobson et al. 2013), while in patients suffering from angina, the QTVI has been found to correlate with the extent of coronary artery disease (Balaji et al. 2002). The quotient also increases in ischemic or nonischemic dilated cardiomyopathy (values around -0.43) (Dobson et al. 2013), in hypertrophic cardiomyopathy (about 50 % rise of baseline values) (Berger 2003), as well as among people resuscitated from cardiac arrest, especially those who had recurrent arrhythmic episodes (Berger 2003).

Interestingly, high QTVI values have also been found in various other diseases such as Kawasaki disease, familial dysautonomia, type 1 myotonic dystrophy, spinal cord lesions, beta-thalassemia, renal disease, obesity, obstructive sleep apnea, schizophrenia, panic disorder, depression, and acute alcohol withdrawal. In these conditions, the QTVI augmentation results from a reduction in HR variance rather than an elevation in QT variance (Dobson et al. 2013; Tereshchenko and Berger 2011).

Although QTVI has gained popularity over the years, its role in arrhythmic risk stratification is rather limited, and its value in ICD or other therapeutic approaches is not well established (Dobson et al. 2013).

Transmural Dispersion of Repolarization, Tp-e Interval, and Tp-e/QT Ratio

The value and utility of Tp-e and Tp-e/QT ratio as noninvasive, clinical markers of torsadogenicity are well established (Gupta et al. 2008). Prolongation of Tp-e interval due to preferential abbreviation of ADP in epicardial or endocardial cells or preferential prolongation of ADP in M cells is found in conditions with increased TdP risk, such as LQTS, SQTS, Brugada syndrome, and also in organic heart diseases (Table 3). Even though the three aforementioned inherited channelopathies differ regarding the length of QT interval – prolonged in LQTS, abbreviated in SQTS, and normal in Brugada syndrome – they are all characterized by an increased TDR which is associated with ventricular arrhythmogenicity and sudden cardiac death (Antzelevitch 2007).

Compared with other markers, Tp-e interval duration and Tp-e/QT ratio are considered strong and useful predictors of torsadogenicity in both congenital and acquired LQTS, where a preferential prolongation of M-cell ADP results in TDR amplification (Antzelevitch 2007). It is now well known that in LQTS, the prolongation of QT interval is not the only or the most important factor for torsadogenesis (Gupta et al. 2008). The arrhythmogenic risk is strongly related to amplification of TDR which often accompanies QT interval prolongation (Antzelevitch 2008). In general, prolongation of Tp-e interval duration over 117 ms correlates with TDR amplification, which represents the electrophysiologic substrate for reentrant circuits and TdP (Staikou et al. 2014). Nevertheless, in patients with LQTS, Tp-e duration is characterized by significant interindividual variability, with values ranging from 110 to 300 ms (Gupta et al. 2008). Notably, the Tp-e interval is found increased in LQTS type 1 but not in LQTS type 2 patients (Antzelevitch 2007; Gupta et al. 2008). On the other hand, the Tp-e/QT ratio measured in left precordial leads is constantly prolonged, with values between 0.24 and 0.34 (mean value of 0.29) (Gupta et al. 2008). In acquired LQTS, the Tp-e/QT ratio as measured in V5 lead represents a more accurate index of torsadogenesis than QTc or QT dispersion, and values over 0.28 are strongly related to TdP risk (Antzelevitch 2005; Gupta et al. 2008).

The markers have also been used for risk assessment in Brugada syndrome, which is characterized by ST elevations or J wave in V1–V3 precordial leads, usually followed by negative T waves (Antzelevitch 2007; Gupta et al. 2008). The elevated ST segment may present with a saddleback or coved morphology, with the later pattern (Brugada type 1) being associated with a higher risk for sudden cardiac death (Gupta et al. 2008). Interestingly, although the QT interval in these patients may be of normal duration, a change in ST configuration in the right precordial leads from the saddleback to coved type is associated with a significant increase in Tp-e and Tp-e/QT values. In these patients, the Tp-e/QT ratio is significantly increased, with a mean value of 0.32 (Gupta et al. 2008). In general, patients with Brugada syndrome and prolonged Tp-e interval are at a higher risk for recurrent arrhythmias (Gupta et al. 2008).

Both Tp-e and Tp-e/QT ratio have been used as predictors of arrhythmogenicity in SQTS, which is characterized by an abnormally abbreviated ventricular

repolarization. In patients with SQTS, apart from the short QT interval, there may be a prolongation of Tp-e interval indicating TDR amplification and high risk of reentry and TdP development (Antzelevitch 2007). Even in cases without prolonged Tp-e intervals (i.e., Tp-e <110 ms), the Tp-e/QT ratio is increased, and this finding is considered more important for arrhythmogenicity than the absolute Tp-e interval duration (Gupta et al. 2008).

The duration of Tp-e interval has been assessed in several other pathological conditions (Table 3). In organic heart diseases, a prolongation of Tp-e interval indicates a high risk of induction or spontaneous initiation of ventricular tachycardia (Gupta et al. 2008). In patients with hypertrophic cardiomyopathy, an increased Tp-e/QT ratio has been related with sudden cardiac death (Antzelevitch 2007; Gupta et al. 2008). In catecholaminergic polymorphic ventricular tachycardia, TDR amplification due to reversed sequence of transmural activation represents the substrate for development of rapid polymorphic ventricular tachycardia and ventricular fibrillation (Antzelevitch 2007). In acquired bradyarrhythmias, Tp-e has been found more reliable than QT or QTc assessment, with values over 117 s being strongly associated with TdP development (Antzelevitch 2007; Gupta et al. 2008). In acute myocardial infarction with ST elevation, both Tp-e and Tp-e/QT ratio are found significantly increased, with mean values of 114.2 ms and 0.28, respectively. Notably, these high values are measured only in the specific ECG leads with ST elevation and not in the rest leads (Gupta et al. 2008).

The Tp-e duration and Tp-e/QT ratio are also helpful in assessing the torsadogenic potency of various drugs and are considered more important and accurate markers than QT interval duration. It is now well known that drugs which prolong the QT interval are not necessarily torsadogenic. Characteristically, amiodarone and pentobarbital sodium prolong the QT interval, but reduce the Tp-e/QT ratio; these two drugs are not associated with significant risk of TdP (Antzelevitch 2007; Gupta et al. 2008). It has been found that chronic administration of amiodarone prolongs preferentially the APD in epicardial and endocardial cells rather than in M cells resulting in a TDR decrease. Similarly, sodium pentobarbital produces a dose-dependent prolongation of QT interval, but decreases the TDR. Furthermore, cisapride may induce TdP only at concentrations producing maximum TDR increase (0.2 μmol/L) but not at higher concentrations which are associated with maximum QT prolongation (Antzelevitch 2005).

T Wave Alternans

Useful information can be derived from MTWA analysis regarding arrhythmogenicity in certain pathological conditions (Table 3). High values are generally associated with increased incidence of cardiovascular mortality and sudden cardiac death (Verrier et al. 2011). The magnitude of alterations is increased in tachycardia, myocardial ischemia, heart failure, and sympathetic activation, reflecting the amplification of cardiac electrical instability in these conditions (Verrier et al. 2011). Conversely, TWA decreases in the presence of beta-adrenergic or sodium channel

blockade, in spinal cord or vagus nerve stimulation, and is associated with reduced vulnerability to dysrhythmias (Verrier et al. 2011). The MTWA may contribute to prediction of sudden cardiac death in hypertrophic cardiomyopathy, whereas it is not validated in atrial fibrillation and its role in LQTS and Brugada syndrome remains unclear (Narayan 2006).

The TWA exhibits a high negative predictive value for arrhythmogenicity in patients with reduced systolic function; negative TWA indicates a lower benefit from ICD therapy. Additionally, reduced mortality has been found after application of ICDs in patients with non-negative TWA (Garcia et al. 2011). However, there is no sufficient evidence to support the use of this marker as a routine tool in guiding treatment of cardiac arrhythmias and especially ICD therapy (Floré and Willems 2012; Verrier et al. 2011).

TRIaD

The concept of TRIaD is gaining more and more credibility in laboratories and experimental studies (Hoffmann and Warner 2006) and seems promising in detecting the arrhythmogenic potential of new drugs, especially when assessed together with QT interval duration. When both TRIaD and QT interval prolongation are present, TdP development is possible, while a combination of TRIaD with shortened QT is associated with a high risk of ventricular fibrillation. Interestingly, if QT prolongation occurs without TRIaD, it may actually have an antiarrhythmic effect (Hondeghem 2006; Shah and Hondeghem 2005).

There is no drug described as torsadogenic with negative TRIaD (Hondeghem 2006, 2008), whereas TdP may occur even in the absence of QT prolongation. Thus, the TRIaD concept may probably be useful in preventing the incrimination of chemical entities and their exclusion from clinical practice solely based on QT prolongation.

Beat-to-Beat Variability of Repolarization Duration

The concept of BVR has been introduced in clinical practice as a biomarker of proarrhythmia; it may be helpful in distinguishing patients at risk of ventricular arrhythmias and also drugs with arrhythmogenic potency.

A beat-to-beat modification of repolarization has been noted in individuals with congenital LQTS who are more likely to develop malignant arrhythmias and in patients with nonischemic congestive heart failure susceptible to sudden cardiac death, but without increased QT interval (Table 3). High values of STV have also been associated with arrhythmogenesis in patients with cardiomyopathy and ICDs (Floré and Willems 2012; Varkevisser et al. 2012). In addition, STV elevations are found in patients receiving anthracycline-based chemotherapy, but also in healthy individuals with hypertrophied hearts playing soccer professionally (Varkevisser et al. 2012).

The marker is also used in the assessment of chemical agents' torsadogenic profile; specific drugs, useful in everyday practice, are being tested upon animal models, and their influence on STV is investigated (Oosterhoff et al. 2007; Varkevisser et al. 2012). Drug safety is indicated by absence of changes in STV, despite prolongation of QT interval (Oosterhoff et al. 2007; Thomsen et al. 2006), while arrhythmogenic potency is associated with high values of STV (Varkevisser et al. 2012).

The exact mechanisms underlying the BVR and STV are not, yet, fully understood, and separate software is needed for their calculation in ECG (Thomsen et al. 2006; Varkevisser et al. 2012). Further limitation is the need for 2 min ECG recording for reliable measurement of STV, which is a more time-consuming procedure than a usual ECG recording. Nevertheless, these parameters seem to reflect arrhythmogenic susceptibility better than QT interval prolongation, so they can complement other repolarization markers in proarrhythmic risk assessment (Thomsen et al. 2006; Varkevisser et al. 2012).

Summary Points

- Torsades de pointes is a polymorphic ventricular tachycardia which may progress to ventricular fibrillation and sudden cardiac death.
- The electrocardiographic parameters described below are used as noninvasive markers of torsadogenicity/sudden cardiac death in arrhythmogenic syndromes and heart diseases and also in assessing the safety profile of new drugs.
- QT interval is measured from the QRS start to T wave end and corresponds to the total duration of depolarization and repolarization.
- JT interval (QT–QRS) reflects more accurately the duration of repolarization when depolarization time is prolonged, as in cases with ventricular conduction defects.
- QT dispersion expresses the inter-lead QT variation and is defined as the difference between the maximum and minimum values of QT interval duration.
- QT variability index combines the fluctuations of QT interval and HR in one formula and provides information about the beat-to-beat repolarization lability.
- Tp-e interval and Tp-e/QT ratio are significant torsadogenic markers as they may reflect the transmural dispersion of repolarization.
- T wave alternans expresses beat-to-beat fluctuations in the morphology and amplitude of T wave and ST segment, thus reflecting changes in ventricular repolarization.
- The concept of TRIaD includes the following components: triangulation of the action potential contour, reverse use dependence, instability of action potential duration, and dispersion of repolarization.
- Beat-to-beat variability of repolarization reflects the temporal variability in duration of repolarization among a number of consecutive heartbeats.

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J Wave and Fragmented QRS Formation as a Biomarker

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Masato Shimizu and Mitsuhiro Nishizaki

Contents

Abstract

The presence of the J wave and fragmented QRS (fQRS) in 12 lead electrocardiography has been known for decades. However, their significance for cardiac prognosis and/or arrhythmogenesis has only recently been recognized. For example, dynamism and transfiguration of the J wave is observed in some organic heart diseases including myocardial infarction or vasospastic angina,

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_26

or in idiopathic heart diseases including Brugada syndrome. Thus, the J wave is becoming of increasing interest as a biomarker for cardiovascular disease. Nevertheless, transfiguration of the fQRS has been reported in a few studies of takotsubo cardiomyopathy and acute myocardial infarction. Herein, the historical course of the J wave and fQRS are reviewed, with particular focus on their potential as dynamic and transfigurable biomarkers, as well as application to other diseases.

Keywords

J wave • Fragmented QRS • Biomarker • Idiopathic heart disease • Organic heart disease

Abbreviations

AMI	Acute myocardial infarction
ARVC	Arrhythmogenetic right ventricular cardiomyopathy
CKMB	Creatine kinase MB isozyme
DCM	Dilated cardiomyopathy
ECG	Electrocardiography
Endo	Endocardium
Epi	Epicardium
ER	Early repolarization
ERS	Early repolarization syndrome
fQRS	Fragmented QRS
HCM	Hypertrophic cardiomyopathy
Ito	Transient outward current of potassium
LV	Left ventriculum
PCI	Percutaneous coronary intervention
RCA	Right coronary artery
TTC	Takotsubo cardiomyopathy
VAT	Ventricular activation time
VF	Ventricular fibrillation.

Key Facts

Key Facts of J Wave

- The J wave is classified into six types, with each type exhibiting a degree of correlation to LV arrhythmogenicity and cardiac prognosis.
- The J wave can be altered in amplitude by both depolarization abnormalities and repolarization abnormalities.
- In organic diseases including AMI and VSA, the J wave is a good transfigurable biomarker of ischemia severity.
- In nonorganic diseases including Brugada syndrome or ERS, the J wave is a biomarker of arrhythmogenesis and prognosis.

Key Facts of fQRS

- fQRS is formed by unviable myocardial scar and/or fibrosis.
- The unviable fQRS is a biomarker of cardiac prognosis in some organic diseases including AMI, DCM, HCM, and ARVC.
- fQRS can transfigure due to a depolarization abnormality, undeterminable low-grade fibrosis, or a small ischemic event in the conduction system.
- In TTC, fQRS is a transfigurable biomarker of myocardial damage.
- In AMI or subarachnoid hemorrhage cases, fQRS transfiguration can occur.

Definitions

Acute myocardial infarction (AMI) A fatal cardiac condition with stopping of coronary artery blood flow, resulting in myocardial necrosis within a few days. The interruption of blood flow usually occurs by sudden rupture of a coronary artery plaque, although severe vasospasm or thrombosis can occasionally induce AMI. A patient with AMI feels sudden chest pain and occasionally severe dyspnea caused by heart failure. Electrocardiography shows ST elevation in the territory of a major coronary artery. Fatal ventricular tachyarrhythmia can occasionally appear, and the patient dies due to the arrhythmia or heart failure.

Brugada syndrome An idiopathic heart disease with characteristic electrocardiogram findings and an increased risk of sudden cardiac death, which was found by Spanish cardiologists Pedro Brugada and Josep Brugada. A mutation in the SCN5A gene is known to lead to a loss of the action potential dome of epicardial areas of the right ventricle, which causes transmural dispersion of repolarization. The transmural dispersion induces characteristic ST elevation in precordial leads, which is termed a coved type or saddle-back type ST elevation.

Early repolarization (ER) An ECG variant concept including J wave and characteristic ST elevation in Brugada syndrome. Historically the term ER was used in all patterns with ST elevation at the J point. At present, ER is used to indicate only the J wave and Brugada type ST elevation. However, the term ER syndrome reflects as syndromes except Brugada syndrome.

Early repolarization syndrome (ERS) An idiopathic heart disease with an ER pattern (= J wave) in the inferior, lateral, or inferolateral leads, which excludes ER in precordial leads (Brugada syndrome). It is sometimes called a J wave syndrome. Historically, ER pattern was considered a benign finding. However, patients who suffered fatal ventricular tachyarrhythmia and sudden death were observed to have ERS, and a definitive association between J waves with early ER pattern and VF was reported.

Fragmented QRS (fQRS) An additional R wave (R′) or notching in the nadir of the S wave or the presence of >1 R fragmentation in two contiguous leads,

corresponding to the territory of a major coronary artery. fQRS is restricted in narrow (<120 ms) QRS cases. However, it is expanded in wide QRS cases or pacing cases. In many heart diseases, the presence of fQRS is known to be associated with cardiac prognosis.

I_{to} A transient outward potassium current across the myocardial cell membrane, which contributes during the repolarizing phase 1 of the action potential. The current results in the movement of potassium (K+) ions from the intracellular to the extracellular space.

J wave Small ST elevation at the last point of QRS wave (J point). At least 1 mm (0.1 mV) above the isoelectric line, either as QRS slurring (smooth transition from the QRS segment to the ST segment) or notching (positive J deflection inscribed on the S wave) in the inferior lead (II, III, and aVF), lateral lead (I, aVL, and V4–V6), or both. J wave is known to relate to left ventricular arrhythmogenicity and cardiac prognosis.

Lambda wave A characteristic ST elevation fuse-merged with slurred type J wave in cordial leads, which appears in acute myocardial ischemia. Fatal ventricular tachyarrhythmia occurs after lambda wave appearance.

Osborn wave The J wave in hypothermic patients, which was found by Osborn in 1953. A relationship between the Osborn wave and ventricular fibrillation was observed. The wave was originally termed a "current of injury" due to acidosis with hypothermia. At present the Osborn wave is termed the "hypothermic J wave".

Takotsubo cardiomyopathy (TTC) Acute non-ischemic cardiomyopathy with sudden onset of chest pain, syncope, and echocardiography and/or left ventriculography shows characteristic apical ballooning and basal hyperkinesis without coronary artery stenosis. The apical asynergy often recovers within a few days, and the prognosis is thought to be relatively good. However, sometimes fatal ventricular tachyarrhythmia or ventricular rapture occurs in acute phase of TTC. The onset is triggered by emotional stress, and the mechanism of apical ballooning is explained by catecholamine toxicity.

Vasospastic angina (VSA) A clinical syndrome with sudden chest pain due to coronary artery vasospasm. The spasm is induced by smooth muscle constriction of the coronary artery, which sometimes leads to myocardial infarction, ventricular tachyarrhythmias, and sudden cardiac death. The syndrome was first reported by Prinzmetal et al. in 1959 in a patient with untypical angina attack. Thus, the syndrome is sometimes termed Prinzmetal angina or variant angina. By acetylcholine provocation test in coronary angiography, the syndrome can be diagnosed with high sensitivity.

Ventricular fibrillation (VF) Fatal life-threatening arrhythmia, which is uncoordinated contraction of the cardiac muscle of the left ventricles, is a major

cause of sudden cardiac death. VF is a medical emergency condition that requires prompt cardio-pulmonary resuscitation including electrical defibrillation.

Introduction

Twelve lead electrocardiography (ECG) was first invented by Einthoven in 1903, and many ECG parameters have since been discovered for evaluation of various heart diseases. However, there are no systematic reviews of the use of ECG parameters as biomarkers for cardiovascular disease. A biomarker generally refers to a measurable indicator of some biological state or condition (The National Institutes of Health (NIH) Definitions Working Group 2000). The NIH defines a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to therapeutic intervention" (Aronson 2005).

To valuate ECG parameters as diamic biomarkers, the parameter should appear, amplify, transfigure, and diminish in proportion to the degree of the cardiac disease. There are many parameters that can be derived from the ECG, some of which have been used as biomarkers. The most famous and well-used ECG biomarker involves ST-T levels in acute myocardial infarction (AMI). For example, in the hyperacute phase of anterior wall AMI, the T wave in the precordial lead shows kurtosis, which is termed a hyperacute T wave (Lane and Holmes 2008). Within several hours, the ST level shows elevation, which is diminished after a few days, while a negative T wave appears. ST-T transfiguration is considered a good biomarker in AMI. Other ECG parameters including Q wave and QT interval also show biomarker-like changes in AMI. Interestingly, many of the classical ECG parameters have also been re-evaluated as biomarkers. For example, ventricular activation time (VAT; an interval between the onset of Q wave to peak of R wave) was first proposed by Lewis in 1913 and then studied up to the 1970s (Lewis 1925; Macleod et al. 1930). Recent research has provided new insight into the use of VAT as a cardiac biomarker. The J wave and fragmented QRS have also been recently evaluated as potential markers of cardiovascular disease.

The J wave is defined as ST elevation at the last point of the QRS wave (J point). The amplitude of the J-point elevation is at least 1 mm (0.1 mV) above the isoelectric line, either as QRS slurring (smooth transition from the QRS segment to the ST segment) or notching (positive J deflection inscribed on the S wave) in the inferior lead (II, III, and aVF), lateral lead (I, aVL, and V4–V6), or both (Fig. 1) (Sato et al. 2012; Antzelevitch and Yan 2010). The presence of the J wave is now known to relate to left ventricular (LV) arrhythmogenicity and cardiac prognosis. The J wave was first described in 1938 by Tomaszewski, who reported an ECG of a man frozen to death (Tomaszewski 1938). A J wave in a hypothermic patient was subsequently reported (Emslie-Smith 1956), and J waves were then frequently reported in normal healthy subjects. In 1936, the prevalence of the J wave in young subjects was examined (Shipley and Hallaran 1936) and reported occurrence rates of 25 % in males and 16 % in females. In that study, J deflection was defined as slurring or

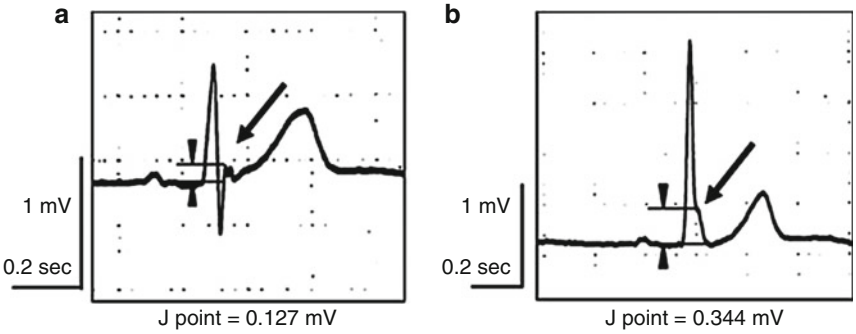

Fig. 1 J wave morphologies. (**a**) A notched type J wave (*arrow*): a positive deflection inscribed on the S wave. The amplitude of the J wave was 0.127 mV. (**b**) A slurred type J wave (*arrow*): a smooth transition from the QRS to the ST segment. The amplitude of the J wave was 0.344 mV. The J wave was defined as positive when the J point was $\geq$ 0.1 mV above the isoelectric line in $\geq$ two contiguous leads (*arrowheads* and *lines*) (From Sato et al. 2012)

notching of the terminal part of the QRS complex. The term "early repolarization" (ER) was first described by Grant et al. in 1951 (Grant et al. 1951), in which the ST-segment deviations and T-wave changes were considered to result from premature repolarization. It was proposed that the J wave was "the normal RS-T segment elevation variant" and defined the ER as onset of the ST segment elevation at the J junction of the QRS, which was altered from its isoelectric line accompanied by downward concavity of the ST segment (Wasserburger and Alt 1961). J waves were also examined in 65 cases (maximum age of 26 years) and concluded that the J wave was a benign finding (Kambara and Phillips 1976). The first historical report correlating the J wave to ventricular tachyarrhythmia was described by Osborn in 1953 (Osborn 1953). In that study of hypothermic canines, ventricular fibrillation (VF) occurred in all animals that had a J wave on their ECG, and the authors suggested a "current of injury" due to acidosis and hypothermia that fibrillated at rectal temperatures less than 25 °C. This "current of injury" was later termed the "Osborn wave."

In 1992, it was reported that cases with idiopathic VF sometimes exhibit J waves (Aizawa et al. 1992). More recently, a definitive association between J waves with an early ER pattern and VF was found (Haissaguerre et al. 2008), and the J wave was suggested an important indicator for risk for sudden death, termed "early repolarization syndrome" (ERS). The ERS and J wave were subsequently classified into inherited type and acquired type (Table 1) (Antzelevitch and Yan 2010). The ERS was reclassified into lateral ER, inferolateral ER, widely ER, and Brugada syndrome, while the J wave was reclassified into ischemic ER and hypothermic ER. The types of J wave and their characteristics of arrhythmogenesis and prognosis have been widely studied. However, there are no reports of the J wave as a "dinamic biomarker," an indicator of biological and pathogenic processes, or pharmacological responses for a disease. The longitudinal changes in the J wave were examined in 542 patients with J wave on baseline ECG, with repeated ECG an average of 5 years

Table 1 Classification of J wave (Early repolarization)

	Inherited				Accuired	
	Lateral leads J wave	Inferior or inferolateral leads J wave	Grobal J wave	Brugada syndrome	Ischemia mediated J wave	Hypothermia medicated J wave
J wave	I, V4-6	II, III, aVF	Grobal leads	V1-3	Any leads	Any leads
VT/VF	Rare	Yes	Yes, and electrical storm	Yes	Yes	Yes
Sex dominance	Male	Male	Male	Male	Male	Either
Response to Na chanel blocker	↓→	↓→	↓→	↑	NA	NA
Response to quinidine						
J wave	↓	↓	↓	↓	Limited Data	
VT/VF	↓	↓	↓	↓	Limited Data	↓
Response to isoproterenol						
J wave	↓	↓	Limited Data	↓	NA	NA
VT/VF	↓	↓	Limited Data	↓	NA	NA

Classification of J wave and each characteristics. *ER* Early repolarization, *VT/VF* Ventricular tachycardia/ventricular fibrillation (Antzelevitch and Yan 2010, with modification)

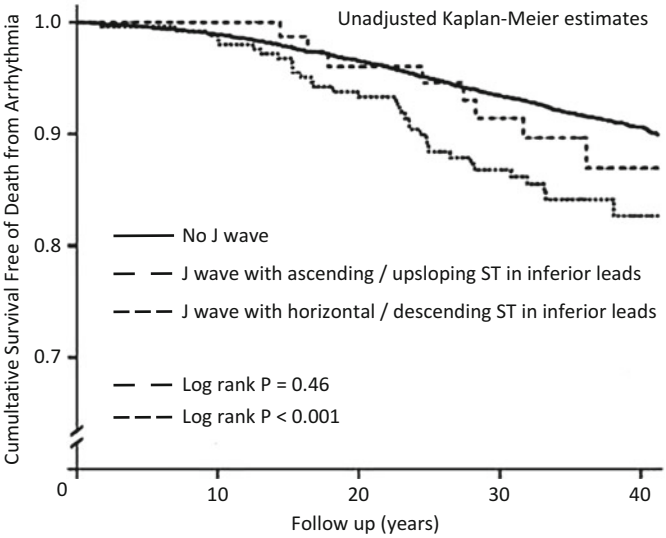

Fig. 2 Long term survival free of death from arrhythmia demonstrated by Tikkanen et al. The *vertical axis* shows survival rate, and the *horizontal axis* shows survival years. The *solid line* shows subjects without a J wave, and the *rough block line* shows subjects with a J wave following an ascending/upsloping ST segment. The *tight block line* shows subjects with a J wave following a horizontal/descending ST segment (From Tikkanen et al. 2011, with modification)

after baseline (Tikkanen et al. 2009). In that study, the J wave disappeared in 99 of the subjects (18.3 %). The long-term prognosis associated with the J wave was also examined in 10,132 subjects, 90 of whom exhibited a J wave with ascending/upsloping ST segment and 265 with a J wave with horizontal/descending ST segments; the other 9777 subjects had no J wave (Tikkanen et al. 2011). The long-term survival free of death from arrhythmia and its Kaplan–Meier estimates for these subjects are shown in Fig. 2. Subjects with a J wave with horizontal/descending ST segment had significantly worse prognosis than those with no J wave. The relationship between the J wave and serum testosterone levels was also reported (Haruta et al. 2011). Testosterone levels peak in the 20s in males and then decrease in proportion to age. The testosterone level is a changeable biomarker for males; however, the transfiguration of the J wave over the human life span with respect to cardiac events is unknown. Nevertheless, over the short clinical time course, the significance of the J wave as a dynamic biomarker had been determined in some cardiac diseases, which are described in the following chapter.

Fragmented QRS (fQRS) is a relatively new concept, which is defined as an additional R wave (R′), notching in the nadir of the S wave, or the presence of >1 R fragmentation in two contiguous leads, corresponding to the territory of a major coronary artery in narrow (<120 ms) QRS cases (Fig. 3) (Das et al. 2007). The presence of fQRS is now known to be associated with cardiac prognosis in cases with organic heart diseases (Hayashi et al. 2013). Historically, QRS slurring was first

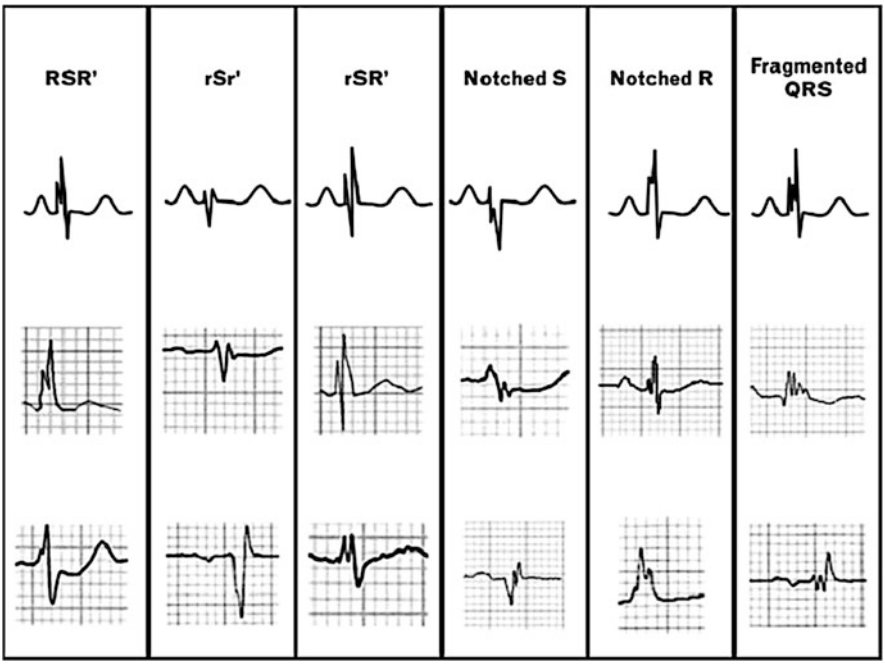

Fig. 3 The morphological classification of fQRS as described by Das et al. fQRS was originally defined only in a narrow QRS (≤120 ms). The definition of fQRS is now expanded to include wide QRS cases. *fQRS* fragmented QRS (From Das et al. 2007)

reported in 1969, with QRS waves (termed high-frequency components) commonly observed in cases with prior myocardial infarction and ventricular enlargement (Flowers et al. 1969). In 1970, the RSR′ pattern in patients with LV aneurythm was reported (El-Sherif 1970). In 1983, first evidence of a relationship between QRS fragmentation and ventricular tachyarrhythmia was reported (Wiener et al. 1984). By direct recording with an intracardiac catheter, QRS fragmentation of endocardial ECG was found to be related to late potential of signal averaged ECG, which was closely related to fatal ventricular arrhythmia. On body surface ECG, the clinical significance of fQRS has been reported in coronary artery disease (Das et al. 2006), arrhythmogenetic right ventricular cardiomyopathy (ARVC) (Peters et al. 2008), and Brugada syndrome (Morita et al. 2008). Further, it was demonstrated that fQRS was a predictor of mortality and sudden cardiac death in acute coronary syndrome (Das et al. 2007). The concept of fQRS has been expanded to wide QRS and/or paced QRS (Das and Masry 2010). For example, Hayashi et al. examined 98 patients with organic heart disease and demonstrated that the prognosis of patients with fQRS was significantly worse than that of patients without fQRS (Fig. 4) (Hayashi et al. 2013). Overall, these studies suggest a potential involvement of fQRS in cardiovascular disease. However, there is a new study showing a role for fQRS study as a transfigurable myocardial damage marker. The cases were demonstrated with

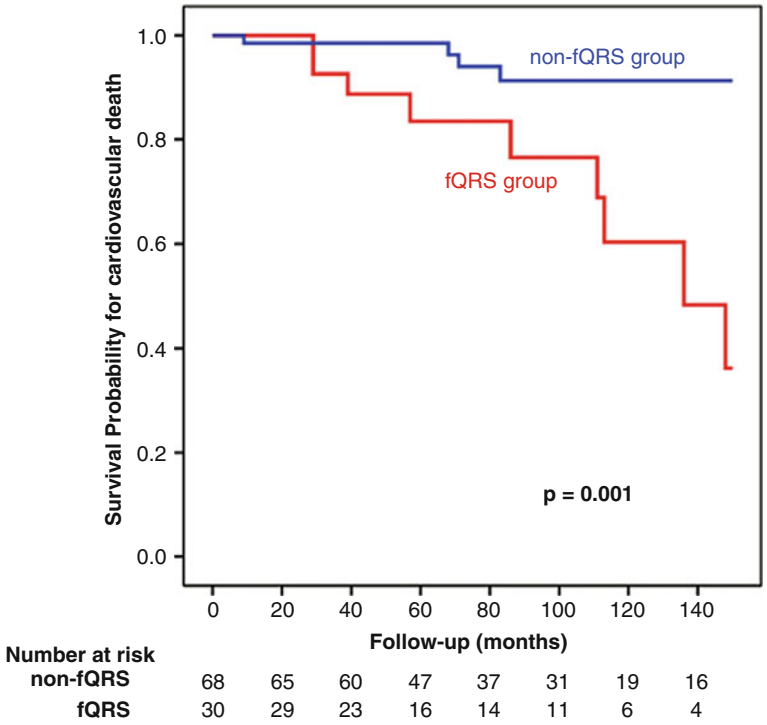

Fig. 4 Kaplan–Meier curve showing survival from all cause of death in patients with and without fQRS. The *red line* shows patients with fQRS. The *blue line* shows patients without fQRS. *fQRS* fragmented QRS (From Hayashi et al. 2013)

appearance of the fQRS only in hyperacute phase of takotsubo cardiomyopathy, which is then diminished or disappears within 48 h after disease onset (Shimizu et al. 2014). Alternatively, fQRS has been suggested to be induced by unviable dead myocardial scar and/or fibrosis.

The aim of this chapter is to describe the mechanisms of J wave and fQRS formation and transfiguration, and to demonstrate their significance as biomarkers in several cardiac diseases.

The Mechanisms of J Wave Formation and its Transfiguration

The molecular basis of J wave formation was elucidated by Gussal and colleagues in 2000 (Gussak and Antzelevitch 2000). In that study, an ER pattern was observed in a canine coronary-perfused wedge preparation model, which could readily convert to phase 1 in which phase 2 reentry gave rise to polymorphic ventricular tachyarrhythmia. The mechanism of J wave formation was suggested to involve differences in the action potential form between the epicardium (Epi) and endocardium (Endo), as

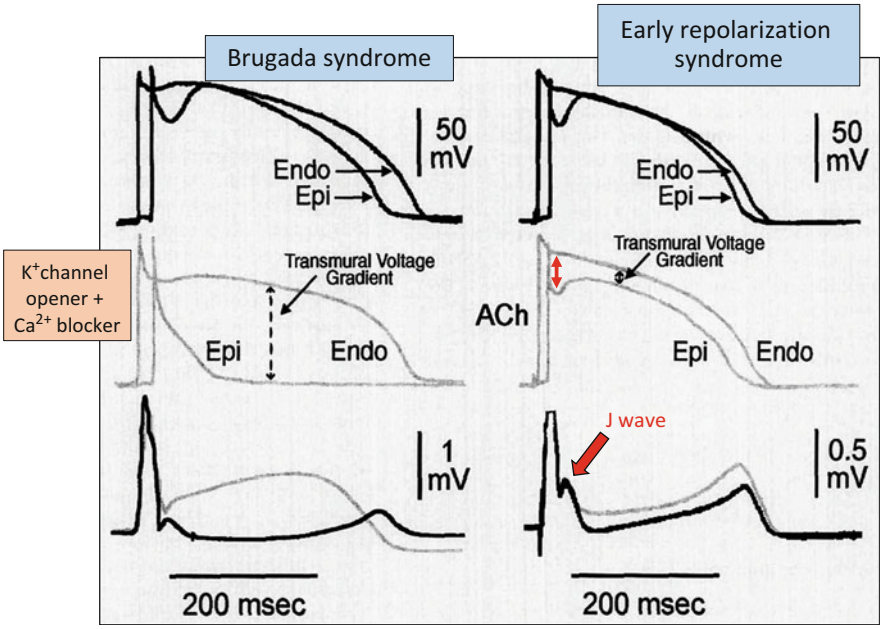

Fig. 5 The molecular mechanisms of ER. *Left side* shows Brugada syndrome. *Right side* shows ERS. *Upper figure* shows action potential sequence of the epicardium and endocardium. *Middle figure* shows the change of action potential by K^+ channel opener or Ca^{2+} blocker. The *lower figure* shows the ECG pattern at rest and with drug treatment (From Gussak and Antzelevitch 2000, with modification)

observed for Brugada syndrome (Fig. 5). The ventricular Epi action potential displayed a prominent transient outward current (I_{to}), which formed the first phase of transmembranous potential with a higher action potential in Epi than in Endo. Therefore, in the epicardial site, I_{to} produces a deep notch between the first and second phase, while the membrane potential produces the spike and dome form. Thus, the positive ECG wave was the result of differences between the Epi and Endo action potential waveforms. Compared with normal repolarization, early repolarization models show a sharp form of the positive wave, and a sharp J wave is recorded. In Brugada syndrome, the marked shortening of action potential duration results in marked ST elevation and concealing of the J wave. Conversely in ERS, the plateau of action potential in Epi is inhibited, which induces a relatively small ST elevation, and a marked J wave is therefore recorded in ERS. In summary, the shape and amplitude of the J wave depends on (1) the transmural distribution of the action potential notch amplitude, (2) the relative time course of the early phases of the action potential (width of notch) at different sites within the wall, (3) sequence of activation, and (4) conduction time across the wall.

The mechanisms of J wave formation have been suggested to involve both depolarization and repolarization abnormalities. However, the predominant

mechanism for use of the J wave as a biomarker remains controversial. In a study of a human case with a J wave in the V4 lead, an electrode was inserted into the LV myocardium at 36 points, and the J wave was found to be formed within the repolarization period (Boineau 2007). Conversely, patients with both J wave and existence of LV pseudotendon were examined and revealed that the pseudotendon was closely related to J wave formation, which induced a delayed conduction through the pseudotendon (depolarization abnormality) associated with J wave formation (Nakagawa et al. 2012). Further, the circadian variation of the J wave was examined in 22 cases of idiopathic VF and concluded that J waves were more closely associated with a depolarization abnormality rather than repolarization abnormality (Abe et al. 2010). Importantly, the amplitude of the J waves is altered by both depolarization and repolarization abnormalities, and changes in proportion to the severity of the disease, providing support for use of the J wave as a dynamic biomarker.

The Mechanisms of fQRS Formation and its Transfiguration

Although the exact mechanism of fQRS formation and its pathophysiology are not fully elucidated, a number of mechanisms have been proposed. Hayashi et al. suggested a role for conduction block theory, whereby the scaring or ischemic myocardial tissue causes localized conduction blocks, leading to additional R′, notching of the S wave, or notching of the R wave (Hayashi et al. 2013). Das et al. also reported that fQRS had a higher sensitivity and negative predictive value compared with the Q-wave in prior MI cases, suggesting that different fQRS patterns might represent various directions of the myocardial activation pattern, depending on the extent and location of ventricular scar tissue (Das et al. 2006). In a study of nonischemic dilated cardiomyopathy, Basaran et al. reported a role for dyssynchrony theory as a mechanism of fQRS formation (Basaran et al. 2011). In support of this mechanism, myocardial scar and/or ischemia was reported to cause heterogeneous ventricular activation resulting in ECG fragmentation (Hayashi et al. 2013; Friedman et al. 1975). Further, fQRS complexes and dyssynchronous contraction patterns were suggested to occur secondary to heterogeneous intraventricular activation and uncoordinated depolarization of viable myocyte groups that are surrounded by fibrotic tissue (Pietrasik et al. 2007).

Pathophysiologically, fQRS tends to be untransfigurable, such as for an old Q wave. Further, the fQRS was largely considered to be induced by regional myocardial fibrosis and/or scarring, which have no tissue viability. However, there is now increasing evidence for a transfigurable fQRS. For example, it was reported that the fQRS could exist even in the absence of determinable myocardial fibrosis (MacAlpin 2010), and the mechanisms were suggested to involve a depolarization abnormality or undeterminable low-grade fibrosis. It was also examined whether QRS fragmentations could identify patients with increased systemic inflammatory status and suggested a role for transfigurable fQRS involving focal fibrosis and ischemia within the conduction system (Cetin et al. 2012). As for the J wave, the changes in fQRS

amplitude are important when considering its use as a biomarker. Compared with the J wave, the difficulty in evaluating the fQRS as a transfigurable biomarker might relate to difficulty in evaluating its morphology. Using an automated algorithm to examine fQRS morphologies, Maheshwari et al. established an evaluation system with high sensitivity and specificity (0.897 and 0.899, respectively) (Maheshiwari et al. 2013). Using this system, the significance of fQRS as a biomarker in many cardiac diseases has since been evaluated.

J Wave and fQRS in Organic Heart Diseases as a Biomarker

The use of the J wave and fQRS as dynamic biomarkers in cases of takotsubo cardiomyopathy (TTC) has previously been reported (Shimizu et al. 2014). TTC can cause serious cardiac conditions including life-threatening ventricular arrhythmia. Thirty-one consecutive patients with TTC were examined; from these, nine patients (29 %) were found with J waves and/or fQRS (Fig. 6a, b). The J wave and fQRS appeared only in the hyperacute phase and were diminished or disappeared immediately in proportion to the degree of TTC. In these patients, the left ventricular ejection fraction was significantly lower, and the summed defect score of single-photon emission computed tomography using iodine 123 beta-methyl-*p*-iodophenyl-pentadecanoic acid and creatine kinase MB isozyme (CKMB) were significantly higher, than patients without J wave or fQRS. Multivariate analysis also indicated that CKMB was a significant indicator of J wave or fQRS appearance (Table 2). Further, the J wave was a significant indicator for cardiac death and/or ventricular tachyarrhythmia (odds ratio 11.5; $P = 0.026$) (Table 3). Recently, TTC induction has been explained by catecholamine toxicity theory (Akashi et al. 2008), whereby toxicity might induce a mismatch leading to loss of the action potential dome between the epicardium and the endocardium, which induces the J wave. Although there is no suitable biomarker for catecholamine toxicity in the myocardium, the J wave is considered a good biomarker of TTC.

There is very little evidence for a role of J wave and/or fQRS as biomarkers in other organic heart diseases. However, the J wave and fQRS have been reported to relate myocardial damage, arrhythmogenicity, or prognosis. For example, J waves and fQRS have been widely studied in acute myocardial infarction. The typical "ischemic J wave" is observed in acute myocardial infarction following right coronary artery (RCA) occlusion, and occlusion of the conus branch of the RCA was reported to induce J wave and ST elevation (coved-type elevation) in precordial leads, similar to that observed for Brugada syndrome (Nakazato et al. 2000). In AMI due to left coronary artery occlusion, the "lambda wave" of the J wave was reported, which involved a slurred-type J wave fused with an elevated ST segment in cordial leads (Gussak et al. 2004). It was also reported that the mechanism of lambda wave formation involved merging of the amplified J wave and elevated ST segment (Gussak et al. 2004). The lambda wave is considered an important biomarker for fatal arrhythmia. Although there are few reports of a dynamic J wave in AMI, an interesting out-of-hospital cardiac arrest AMI case was described, in which

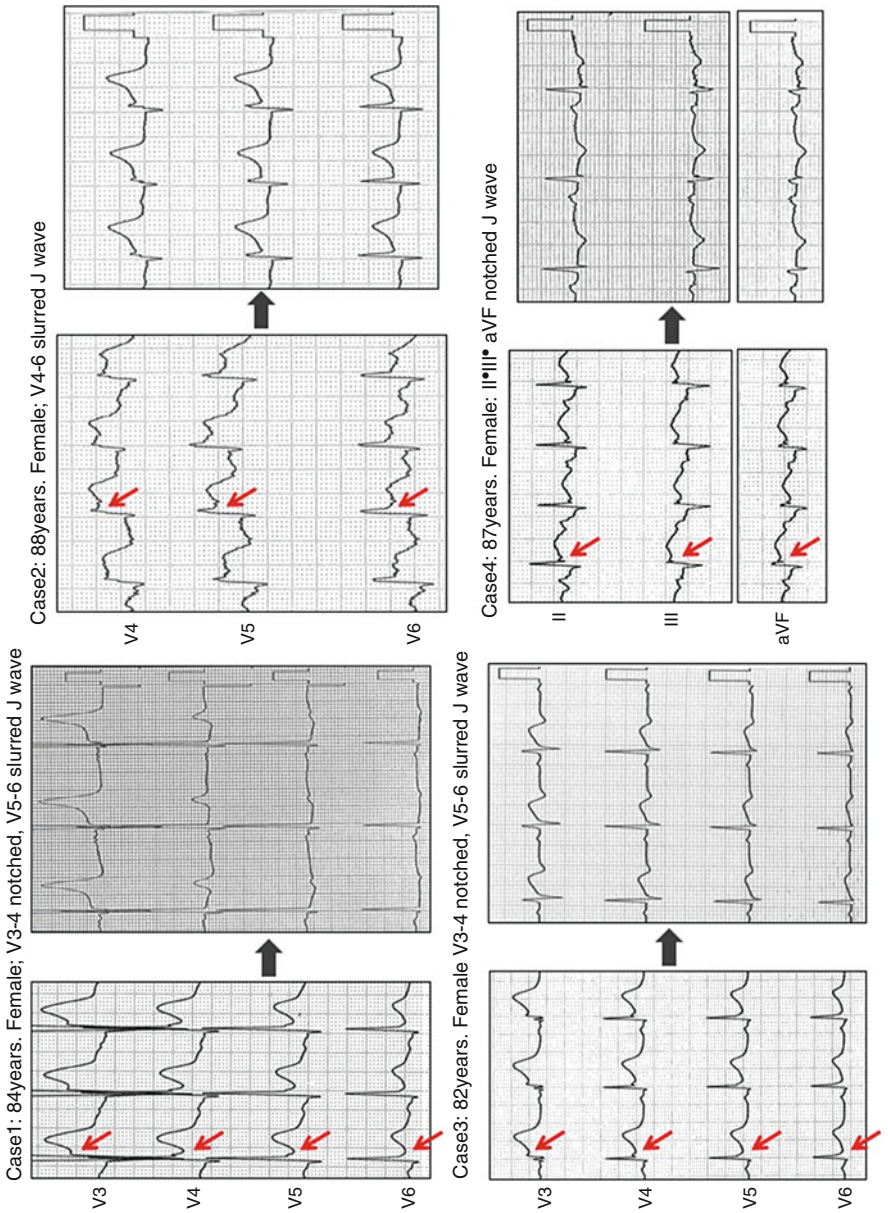

Fig. 6 (continued)

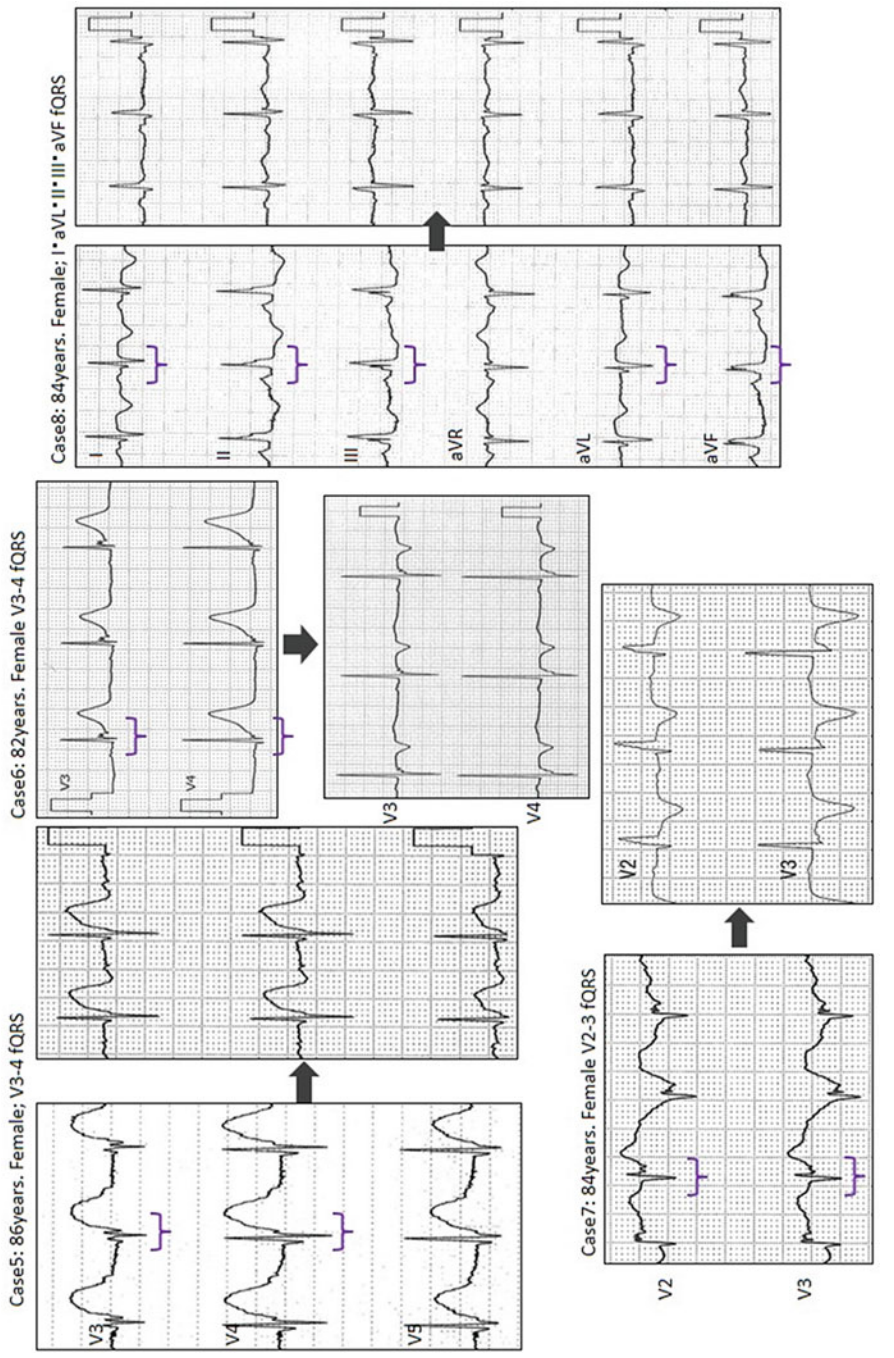

Fig. 6 (continued)

Table 2 Multivariate analysis for hyperacute J wave or fQRS in takotsubo cardiomyopathy

	Univariate		Multivariate (stepwise regression)	
	P value	OR (95 % CI)	P value	OR (95 % CI)
Age	0.045*	1.39 (1.01-1.91)		
Ejection Fraction	0.032*	0.86 (0.75 0.99)		
Max CKMB	0.008*	1.06 (1.02–1.10)	0.045*	1.11 (1.00-1.22)
SRS BMIPP	0.037*	1.19 (1.01–1.40)		
SRS TL	0.133	1.13 (0.95–1.33)		
Heart Rate	0.125	1.03 (0.99–1.08)		
QTc	0.129	0.99 (0.97–1.00)		
Inverted T wave	0.005*	0.04 (0.00–0.37)		
VT or VF	0.336	2.86 (0.34–24.3)		
PVC	0.936	1.00 (0.99–1.01)		
ST elevation	0.017*	9.33 (1.50–58.2)		
All causes of death	0.116	5.00 (0.67–37.3)		
Cardiac death	0.559	1.81 (0.25–13.2)		

Univariate analysis and multivariate stepwise logistic regression analysis for appearance of J wave or fQRS. Heart Rate, QTc, and Inverted T wave were evaluated at admission. *$P < 0.05$ was considered statistically significant. *CKMB* creatine kinase MB isozyme, *SRS* summed rest score, *BMIPP* iodine-123 beta-methyl-iodophenylpentadecanoic acid, *TL* Thallium-201, *VF* ventricular fibrillation, *VT* ventricular tachycardia, *PVC* premature ventricular contraction, *OR* odds ratio, *95 % CI* 95 % confidence interval (Shimizu et al. 2014)

prominent ischemic J waves were documented during recurrent VF attacks (Myojo et al. 2012). In that study, ECG after the first cardioversion did not have J waves, but during repeated VF, J waves frequently appeared in the inferior leads. Thus, the appearance of J waves was suggested to be an important marker for lethal arrhythmias in acute ischemia.

Ischemic J waves have been widely reported in vasospastic angina. A case has been reported in which RCA vasospastic angina induced a phenomenon similar to Brugada-like ST elevation in the precordial leads (Ihara et al. 2009). It also demonstrated augmentation of the J wave following VF in VSA patients by acetylcholine provocation test (Sato et al. 2012), where J wave amplitude was altered in proportion to the severity of spasm (Fig. 7). Oh et al. reported the presence of the J wave in 20 % of VSA patients, which was associated with serious cardiac events (Oh et al. 2013). Further, they demonstrated dynamic change of the J wave during the clinical course of VSA. Overall, these studies suggest that the J wave in VSA might be a useful biomarker to indicate angina attack and cardiac prognosis including ventricular tachyarrhythmia.

Fig. 6 Cases of J wave and fQRS during the hyperacute phase of TTC. All J waves and fQRS were diminished or disappeared within 48 h from the onset of TTC. (**a**) J wave cases. (**b**) fQRS cases. The presence of the J wave and fQRS predicted myocardial damage, and the J wave predicted cardiac prognosis (see Table 2). *fQRS* fragmented QRS, *TTC* takotsubo cardiomyopathy (From Shimizu et al. 2014)

Table 3 Multivariate analysis for cardiac death and/or VT/VF in takotsubo cardiomyopathy

	Lethal group ($n = 6$)	Non lethal group ($n = 25$)	P value	Multivariate analysis (stepwise regression)	
				P value	OR (95 % CI)
Age (years)	77.7 ± 13.6	75.9 ± 9.6	0.387		
Ejection Fraction (%)	35.9 ± 6.0	49.0 ± 9.4	0.005*		
Max CKMB (IU/ml)	41.2 ± 30.7	27.8 ± 22.0	0.198		
SRS BMIPP	30	11.8 ± 9.5			
SRS TL	25	6.9 ± 5.6			
Heart Rate (bpm)	100.8 ± 14.4	79.1 ± 20.7	0.006*		
QTc (msec)	484 ± 85	503 ± 60	0.308		
Inverted T wave, n(%)	2 (33 %)	16 (64 %)	0.172		
ST elevation, n (%)	5 (83 %)	8 (32 %)	0.012*		
J wave, n(%)	3 (50 %)	2 (8 %)	0.012*	0.026*	11.5 (1.33-99.3)
fQRS, n(%)	0 (0 %)	5 (20 %)	0.273		

Welch t-test, kai^2 test, univariate and multivariate stepwise logistic regression analysis for cardiac death and ventricular tachyarrhythmia (tachycardia/fibrillation). Lethal group included the cardiac death cases and/or ventricular tachyarrhythmia cases. SRS was not analysed because the examination was done in only one case of lethal group. Heart Rate, QTc, and Inverted T wave were evaluated at admission. *$P < 0.05$ was considered statistically significant. *VT/VF* ventricular tachycardia/ventricular fibrillation, *CKMB* creatine kinase MB isozyme, *SRS* summed rest score, *BMIPP* iodine-123 beta-methyl-iodophenylpentadecanoic acid, *TL* Thallium-201, *bpm* beats per minute, *fQRS* fragmented QRS, *OR* odds ratio, *95 % CI* 95 % confidence interval (Shimizu et al. (2014))

The existence of fQRS is known as a good marker to indicate myocardial damage and prognosis in various organic cardiac diseases including dilated cardiomyopathy (DCM) (Ahn et al. 2013), hypertrophic cardiomyopathy (HCM) (Nomura et al. 2014), and ARVC (Peters et al. 2008). Although there are many reports examining the relationship between fQRS and organic heart diseases, few studies have suggested use of fQRS as a transfigurable biomarker. The Q wave in AMI was thought to be similar to fQRS, reflecting myocardial damage and scarring. Although new Q wave formation is an important biomarker in AMI, fQRS has not been used. However, new onset of fQRS was examined in 401 consecutive patients with ST elevation MI who received primary PCI (Ozcan et al. 2014). In that study, patients were assigned to two groups according to persistence or new onset of fQRS and absence or resolution of fQRS at 48 h after PCI, and the evolution of fQRS on pre- and post-PCI ECG and their relationship to myocardial reperfusion parameters were examined. The authors found that new onset or persistence of fQRS after primary PCI was significantly associated with myocardial blush grade <3 and peak creatine kinase MB isozyme levels.

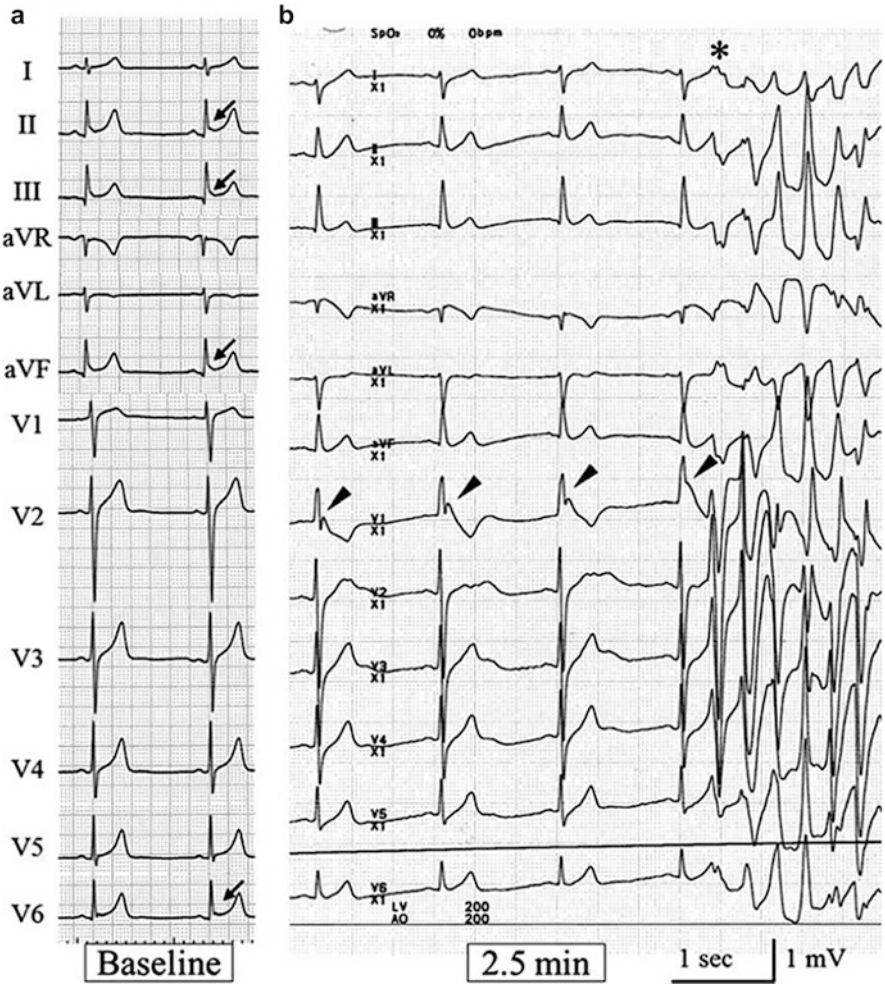

Fig. 7 ECG of a patient with VSA reported by Sato et al. A 43-year-old male patient who underwent an ACh provocation test. (**a**) Baseline ECG showing slur-type J waves with ST-segment elevations in the inferior and V6 leads (*arrows*). (**b**) After ACh provocation into the right coronary artery. The J waves disappeared, and a new J wave appeared in the V1 lead, which was increased in amplitude. A premature ventricular contraction with a close coupling interval (*) and sudden ventricular fibrillation occurred 2.5 min after ACh administration. *ECG* electrocardiography, *VSA* vasospastic angina, *ACh* acetylcholine (From Sato et al. 2012)

The author showed examples of "new onset and persistent fQRS" and "resolutable (vanished) fQRS" (Fig. 8). Figure 8a, b shows the example of the new-onset and persistent fQRS in V2-5 leads in an inferior AMI case. Figure 8c, d shows the example of the resoluble (vanished) fQRS in III and aVF leads in an anterior AMI case. Although they did not compare the two groups directly, the transfigurable fQRS is a biomarker in AMI patients.

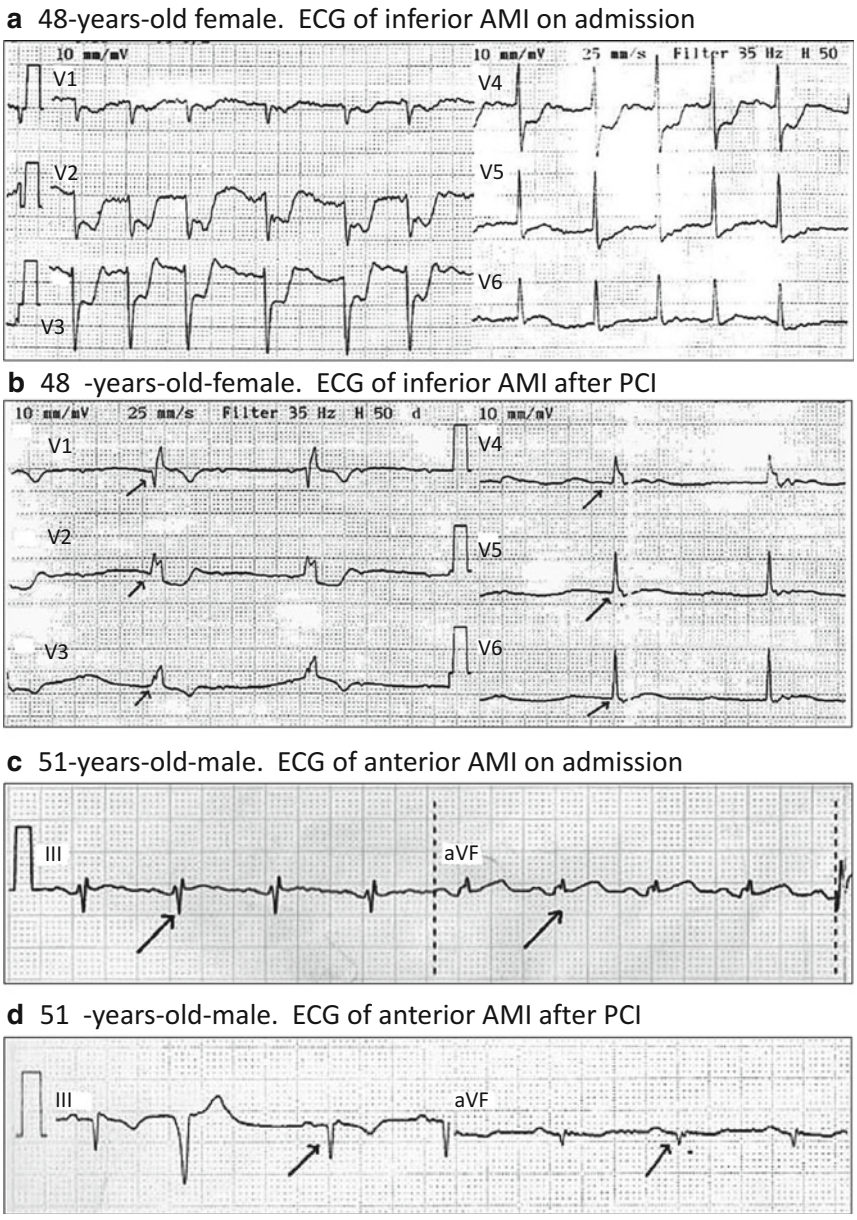

Fig. 8 F-QRS transfiguration in AMI patients. Figure (**a**) and (**b**) shows the example of the new onset and persistent fQRS in V2-5 leads in an inferior AMI case. Figure (**c**) and (**d**) shows the example of the resolutable (vanished) fQRS in III and aVF leads in an anterior AMI case. Althought they did not compared the two groups directly, the transfigurable fQRS is a biomarker in AMI patients. *AMI* acute myocardial infarction, *fQRS* fragmented QRS, *PCI* percutaneous coronary intervention (Ozcan et al. 2014)

J Wave and fQRS in Nonorganic Heart Diseases as a Biomarker

Brugada syndrome is a widely studied nonorganic heart disease that exhibits both ECG biomarkers and J wave appearance. Coved- or saddle back–type ST elevation in the precordial lead is a good biomarker of Brugada syndrome; in particular, the coved-type elevation can fluctuate and indicate occurrence of VF. The J wave in Brugada syndrome had also been studied, although only a few reports have suggested its use as a biomarker for Brugada syndrome. For example, the prevalence of J wave was estimated in inferior and lateral limb leads and the predictive value for the prognosis of Brugada syndrome (Letsas et al. 2008); approximately 12 % of the Brugada syndrome patients had a J wave in the lateral cordial leads, while 8 % patients had episodes of arrhythmia. It was also reported that Brugada syndrome patients with J waves had more cardiac events (syncope or arrhythmia) than the patients without J waves (Sarkozy et al. 2009). Further, it examined the responses to pharmacological provocation to idiopathic VF including Brugada syndrome patients with episodes of VF and found that the J wave was amplified by propranolol and verapamil but diminished by isoproterenol, disopyramide, and cylostazol (Shinohara et al. 2006). As some of these drugs are expected to reduce VF events in patients and are utilized clinically, the J wave may provide a good "biomarker."

It is well established that the amplitude of the hypothermic J wave increases in proportion to the degree of hypothermia. A strong inverse correlation was found between J wave amplitude and body temperature (Fig. 9) (Omar and Camporesi 2014). It was reported that the mechanism of hypothermic J wave transfiguration involved induction of the prolonged plateau phase and enhancement of the notch in the first phase by hypothermia, with inability to maintain the second phase of action potential (Yan and Antzelevitch 1996). It was also reported that the duration of action potential was prolonged in both the Epi and Endo in hypothermia and that the notch made by I_{to} deepened, while the increase of I_{to} and decrease of I_{in} were enhanced, in early repolarizaion (Gussak et al. 1995). As a result, the J wave appeared and was amplified. However, the relationship between cardiac events and J wave remains unclear. Nevertheless, the J wave is likely a good biomarker of hyperthermia.

The significance of the J wave in other nonorganic heart diseases is poorly understood. In short QT syndrome, Watanabe et al. reported a high prevalence of J wave in short QT patients with arrhythmic events (Watanabe et al. 2010). In ERS itself, a case was reported in which J wave amplitude correlated with ventricular arrhythmia, and the J wave was reduced by quinidine provocation, suggesting that J wave amplitude may be an important indicator of therapeutic drug levels and arrhythmia susceptibility in ERS (Sacher et al. 2014).

The fQRS is known to appear in patients with organic heart diseases. However, in Brugada syndrome, It was reported that fragmented QRS appeared to be a marker for spontaneous VF and could predict patients at high risk of syncope (Morita et al. 2008). The authors also suggested that the mechanism of fQRS in Brugada syndrome may relate to conduction delays. In high-risk Brugada syndrome, delayed epicardial activation was reported to cause late potentials, especially during VF

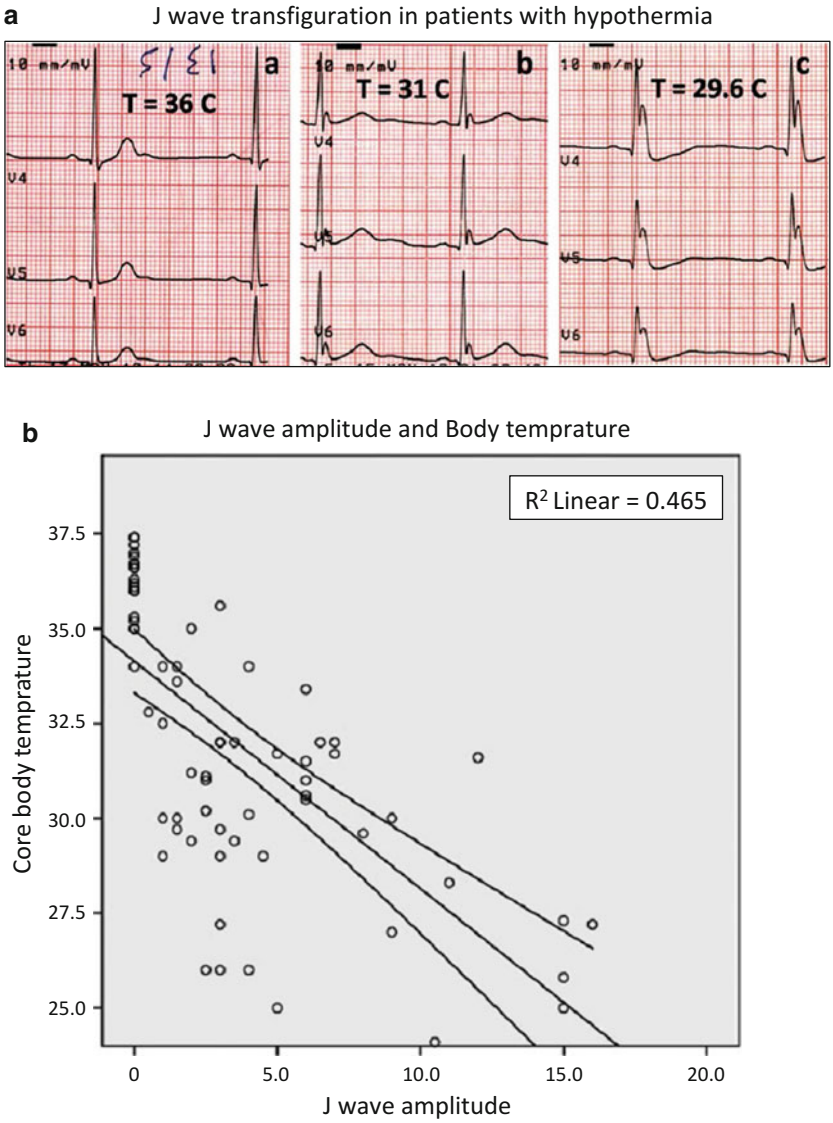

Fig. 9 Correlation between J wave amplitude and body temperature described by Moar et al. (**a**, *upper figure*) Representative ECG of hypothermic patients. V4-6 leads are shown, and a marked notched type J wave was increased in proportion to degree of hypothermia. (**b**, *lower figure*) Relationship between J wave amplitude and core body temperature. The highest J wave in the cordial leads was evaluated. Both had a significant negative correlation ($r = 0.410$, $p < 0.001$) (From Omar et al. 2014)

induction by programmed stimulation in the right ventricular outflow tract (Morita et al. 2008). Although delayed activation within a small part of the myocardium could produce late potentials without significant effects on the QRS, delayed

activation in a larger part of the myocardium could cause multiple spikes within the QRS, resulting in fQRS. Although transfiguration of the fQRS in Brugada syndrome was not found, the increase of delayed epicardial activation may indicate an increased risk of fatal arrhythmia. Thus, fQRS may be a good biomarker in Brugada syndrome. In other nonorganic heart diseases, the significance of the fQRS is unknown, and further studies are required to determine its role as a biomarker.

Potential Applications to Prognosis, Other Diseases or Conditions

J Wave and fQRS in Neurological or Psychological Diseases

As well as a role for the J wave and fQRS as biomarkers for myocardial damage, prognosis, and/or arrhythmogenicity, there is potential for use in other cardiac and noncardiac conditions. For example, there is evidence that they can be used a "stress markers." The role of the J wave and fQRS in takotsubo cardiomyopathy (TTC) has previously been described (Shimizu et al. 2014). TTC is known to have close relationship to mental or physiological stress. For example, the J wave is occasionally observed in cases with various brain diseases, including brain or subarachnoid hemorrhage, and brain trauma (Kukla et al. 2012; Inoko et al. 2005). ECG change was also reported in a case with subarachnoid hemorrhage and simultaneous VF, in which obvious J waves are observed (Kukla et al. 2012). Further, in a patient with subarachnoid hemorrhage, the presence of fQRS was observed in the II/III/aVF leads within 10–20 min after rupture of a basilar artery aneurysm (Inoko et al. 2005). Thus, overall these data suggest that fQRS transfiguration might have significance as a biomarker (Fig. 10).

The autonomic nervous system and the J wave are also known to be closely related. For example, the J wave shows circadian rhythm (Miyazaki et al. 2013), and propranolol or isoproterenol can modify the J wave.[54] As the autonomic nervous system is altered in various brain disorders, the J wave may be useful for evaluating the status of such diseases.

J Wave and fQRS in Endocrine and Renal Diseases

The hypothermic J wave was originally proposed to be induced by respiratory acidosis (Osborn 1953), where amplified J wave VF was observed in a hypothermic canine model without artificial respiration, while animals managed with an artificial respirator showed low-amplitude J waves without VF. Human cases with metabolic acidosis, ketoacidosis, and hyperkalemia were also reported to exhibit the J wave (Moulik et al. 2002). Further, the J wave can appear in cases with hypercalcemia, such as in patients with hyperparathyroidisum (Topsakal et al. 2003). Although fQRS can be used to indicate cardiac prognosis, its prognosis in other organ diseases is unknown. However, fQRS was reported to be independently associated with subclinical LV dysfunction in patients with chronic kidney disease and normal

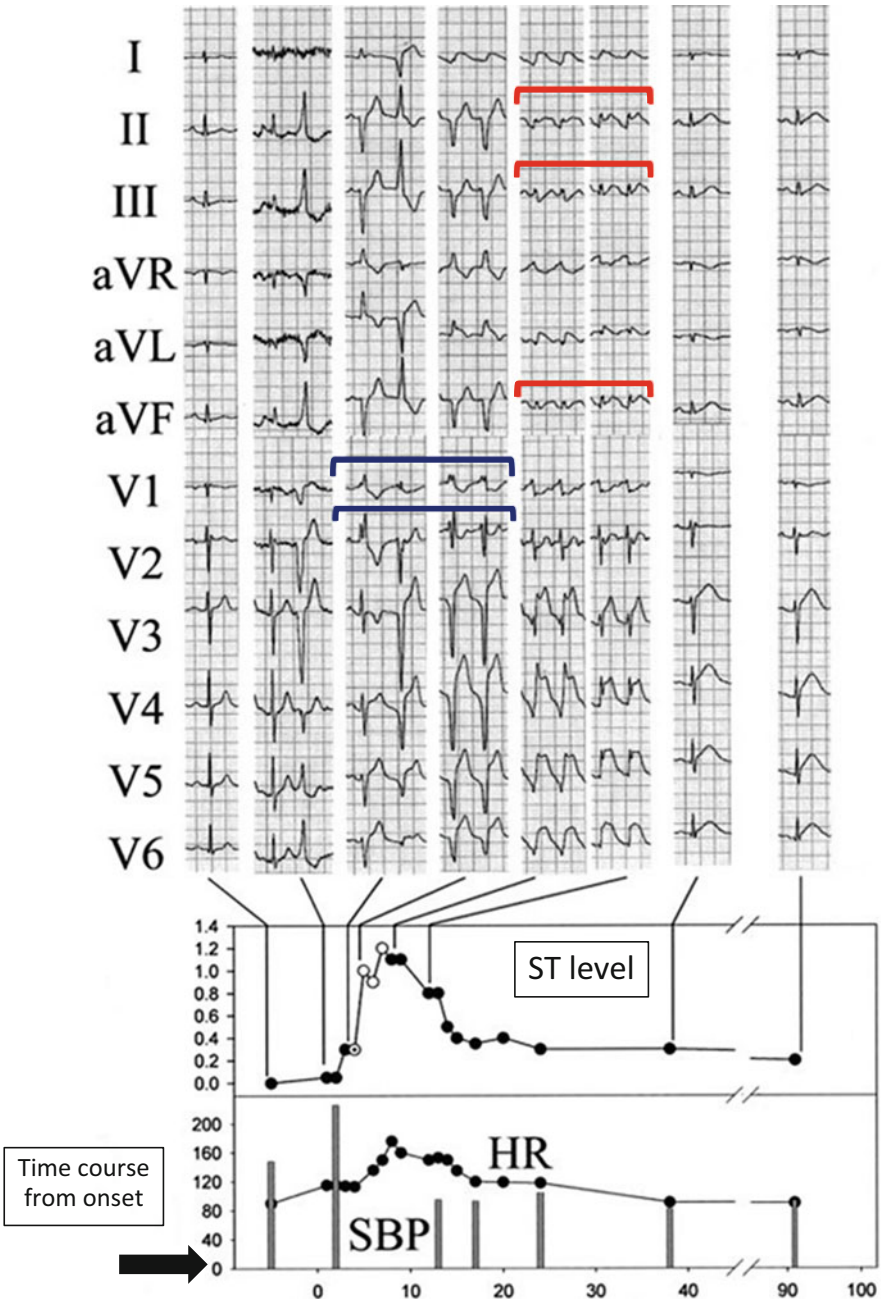

Fig. 10 Serial changes of the ECG and transient fQRS appearance during the progression of subarachnoidal hemorrhage (SAH) reported by Inoko et al. A 69 year old female with no history of hearth disease was treated for an aneurysm at the tip of the basilar artery. During endovascular

ejection fraction (Adar et al. 2014). Thus, fQRS may be useful for predicting prognosis related to cardiac function in other organ diseases.

J Wave and fQRS in Athletes

There is some evidence for the appearance of the J wave in the ECG of athletes. For example, a benign J wave in athletes was reported (Quattrini et al. 2014), while in a study of 21 athletes with sudden cardiac arrest, the presence of J waves was observed in some cases (Cappato et al. 2010). The fQRS may also be useful for determining hypertrophic cardiomyopathy (HCM) in athletes. HCM can be easily diagnosed by other ECG findings and echocardiography, although prediction of prognosis remains difficult. Interestingly, fQRS and T wave inversion suggested in multiple leads represents a high risk of cardiac events in athletes (Zhang et al. 2014). Thus, late potential or T wave alterations, as well as fQRS, may be useful biomarkers for HCM.

fQRS and Rheumatoid Arthritis

fQRS may also be useful as a screening tool in systemic diseases. The fQRS prevalence was reported in patients with rheumatoid arthritis and in control patients with fibromyalgia rheumatica (Kadi et al. 2012). In that study, fQRS was observed more frequently in patients with RA, with disease duration significantly related to the presence of fQRS. Cardiac complications are also observed in other collagen diseases, including polymyositis, scleroderma, lupus erythematodes, and mixed connective tissue disease. Although the appearance of fQRS in these disorders is unknown, F-QRS may have potential as a biomarker of cardiac events or disease prognosis.

Summary Points

- This chapter focuses on the J wave and fQRS as transfigurable biomarkers in many cardiac diseases.
- In this chapter, it was described that the mechanism of J wave formation and its amplitude alters by both depolarization and repolarization abnormalities theories.

Fig. 10 (continued) treatment, the aneurysm ruptured resulting in massive SAH. ECGs were recorded every few minutes, and showed ST elevation and transient fQRS. *Black arrow* shows the time course from the onset of the rupture. *Red parenthesis* shows transient fQRS in II/III/aVF leads. *Purple parenthesis* shows the V1-2 leads. *fQRS* fragmented QRS, *HR* heart rate, *SBP* systolic blood pressure (From Inoko et al. 2005)

- In organic and nonorganic cardiac diseases, the J wave is a good biomarker, with its amplitude altered in proportion to the degree of disease.
- Although fQRS is reported a biomarker due to unviable myocardial scar and/or fibrosis tissues, it may also be useful as a transfigurable biomarker.
- We demonstrated that in some organic diseases including TTC and AMI, fQRS can be a transfigurable biomarker.
- We reviewed the significance of the J wave and fQRS as biomarkers in other diseases including neurological and psychological disorders, endocrine and renal diseases, and collagen and inflammatory diseases.
- The J wave and fQRS may also be useful for cardiac prognosis in athletes.

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Interpretation of Coronary Artery Disease with Intravascular Ultrasound

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Contents

Abstract

The fact that coronary angiography has limitations in terms of precise estimation of atherosclerosis has been partially overcome during the last years by the use of new imaging techniques. Intravascular ultrasound (IVUS) is currently the gold-standard technique for the assessment of the morphology of coronary arteries and atherosclerotic plaques in vivo and an irreplaceable guiding tool for interventional procedures. This chapter summarizes the basic principles along with some newer perspectives of this methodology and evidently highlights not only the use in clinical practice but also the contribution in clinical outcomes.

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_35

Keyword

Atherosclerosis • Coronary angiography • Intravascular ultrasound (IVUS) • Drug eluting stents (DES) • Technical characteristics and detection capability • Basic measurements

Abbreviations

CAD	Coronary artery disease
FFR	Fractional flow reserve
IVUS	Intravascular ultrasound
MI	Myocardial infarction
MLA	Minimum lumen area
NIRS	Near infrared streptoscopy
OCT	Optical computed tomography
RF	Radiofrequency
VH-IVUS	Virtual histology intravascular ultrasound

Key Facts

- Cardiovascular diseases account for approximately more than one third of all deaths worldwide. Atherosclerosis, a disease of the vessel wall, is the major cause of cardiovascular diseases such as heart attack or stroke.
- Most sudden deaths and myocardial infarctions occur from coronary thrombosis caused either by rupture of a thin fibrous cap or surface erosion in the absence of cap disruption.
- The thin-cap fibroatheroma (TCFA) is now regarded as the main type of rupture-prone and thrombosis-prone vulnerable plaque.
- A typical IVUS pullback contains more than 3000 consecutive cross-sectional frames of the examined coronary artery.
- The beneficial impact of intravascular ultrasound (IVUS)-guided versus angiography-guided percutaneous coronary interventions with stent implantation is well established.
- Human coronary atherosclerosis is a dynamic process with potential for the replacement of fibrous tissue by necrotic core. VH-IVUS proved to be beneficial in assessing these intraplaque compositional changes and the outcome of pharmacological treatment.

Dictionary of Terms

IVUS An interventional imaging modality using ultrasound to obtain real time images of the coronary and other vessels of human body.

IVUS pullback The acquisition of an IVUS sequence consists of inserting an ultrasound emitter, carried by a catheter, into the arterial vessel and pulling the probe from the distal to the proximal position (pullback).

Image resolution Transducers with ultrasound frequencies ranging between 20–50 MHz are used (usually 40 MHz). High frequencies provide excellent theoretical resolution, as the ultrasound wavelength that determines the maximum resolution is inversely proportional to frequency.

VH-IVUS A special software that analyzes the unfiltered backscattered IVUS signal mapping with designated colors different tissue components.

Palpography An alternative method of processing the radiofrequency signal for the determination of the elastic properties of plaques that are susceptible to rupture. This method evaluates the local mechanical properties of the tissue using the distortion caused by intraluminal pressure.

CE-IVUS The use of conventional gray scale IVUS with the injection of contrast agents (microbubbles) to record qualitatively and quantitatively the flow of microbubbles in human atherosclerotic plaques, mainly within the microvessels and neovasculature.

VH-TCFA A fibroatheroma without evidence of a fibrous cap: >10 % confluent NC with >30° NC abutting the lumen in at least 3 consecutive frames.

Introduction

In general, pathogenesis of coronary artery disease relates to the slow or rapid and substantial progression of atherosclerosis. Then again, ischemic mechanisms reflect an imbalance between myocardial blood supply and oxygen demand in regard to plaque rupture or superficial erosion with subsequent thrombosis of angiographically mild lesions (vulnerable plaques). The thin-cap fibroatheroma (TCFA), a metabolically active lesion with a large lipid-rich necrotic core and thin fibrous cap, is considered to be the most common type of rupture-prone and thrombosis-prone plaque (Naghavi et al. 2003a, b; Ambrose et al. 1988; Ambrose and Dangas 2000; Fuster et al. 1992a, b; Virmani et al. 2000, 2006; Dangas et al. 1997).

During the past, our knowledge about the genesis, progression, and characterization of atherosclerosis was based mainly on cross-sectional histopathological studies. Coronary angiography has several limitations in assessing plaque burden, calcification, eccentricity, stenosis severity, and also how to implant a stent properly and recognize acute and chronic complications. It is only over the last few years that with the advent of catheter-based devices and techniques which use ultrasound or

optics we are beginning to see beyond angiography (Ambrose and Dangas 2000; Ambrose et al. 1988; Fuster et al. 1992a, b; Virmani et al. 2000).

However, in patients with coronary artery disease, the most important factor with respect to outcome is the presence and extent of inducible ischemia. Treating ischemic lesions improves outcome, but treating nonischemic lesions affects the outcome in a negative way. In patients with stable coronary artery disease, physiologically guided percutaneous coronary intervention (PCI) improves patient outcome as compared with medical therapy alone. In patients with functionally nonsignificant stenoses medical therapy alone resulted in an excellent outcome, regardless of the angiographic appearance of the stenosis (Tonino et al. 2009; De Bruyne et al. 2012).

These are all important clinical aspects that can be addressed in a cath lab setting using invasive assessment of coronary anatomy and physiology.

Intravascular Ultrasound (IVUS)

The first IVUS system was designed in Rotterdam in 1971. It was conceived as an improved technique for the visualization of cardiac chambers and valves. However, the first transluminal images of human arteries were recorded in 1988. Ever since IVUS is useful during stent implantation to assess lesion severity, length, and morphology before stent implantation; to optimize stent expansion, extension, and apposition; and to identify and treat possible complications after stent implantation (Mintz et al. 2001).

IVUS function is based on the general principles summarized in Table 1 (Sanidas et al. 2008). There are two types of IVUS systems for clinical use: the solid-state electronic phased array transducer and the mechanical single-element rotating transducer. The solid-state phased array transducer has 64 stationary transducer elements around the catheter tip that image at 20 MHz, and it is commercially available as the 5 F compatible Eagle Eye Catheter (Volcano Corp. Rancho Cordova, California). Benefits of the solid-state catheter include enhanced trackability due to the coaxial design and lack of nonuniform rotational distortion artifacts seen with rotational systems. Conversely, the 6 F compatible mechanical systems offer a more uniform pullback and greater resolution due to the higher ultrasound frequency. Mechanical systems are available commercially as the 40 MHz iCross or Atlantis SR Pro catheters (Boston Scientific, Santa Clara, California) and the Revolution 45 MHz catheter (Volcano Corp., Rancho Cordova, California) (McDaniel et al. 2011).

Based on studies comparing preprocedural IVUS to flow wire, pressure wire, or nuclear perfusion imaging in terms of clinical outcome, most feel that a lumen area less than 4.0 mm^2 in a proximal epicardial artery excluding left main is a flow-limiting stenosis (Abizaid et al. 1998; Nishioka et al. 1999; Takagi et al. 1999).

The advantage of IVUS guidance has contributed primarily to decreased rates of in-stent restenosis and repeated revascularization in the bare metal stent (BMS) era, mainly by achieving larger acute lumen dimensions while avoiding increased complications (Fitzgerald et al. 2000; Oemrawsingh et al. 2003). The MUSIC trial was

Table 1 General principles of IVUS image acquisition

1	Conversion of electrical energy into sound waves via piezoelectric crystals
2	Transmission and detection of sound waves reflected by tissues using a transducer
4	Conversion of sound waves into electrical energy
4	Amplification and processing of the electrical energy and conversion to an image
5	Projection of that image on the device's computer screen, from where it can be analyzed or stored

Table 2 Optimal stent expansion criteria adopted in the MUSIC study

IVUS criteria defining optimal stent deployment	
1	**Complete apposition of the stent** The stent is apposed against the vessel wall over its entire length
2	**Adequate stent expansion**
2A	MSA $\geq$90 % of the average reference lumen area or MSA $\geq$ 100 % of lumen area of the reference segment with the lowest area when the MSA is $<$9.0 mm^2
2B	MSA $\geq$80 % of the average reference lumen area or MSA $\geq$90 % of lumen area of the reference segment with the lowest lumen area when the MSA is $>$9.0 mm^2
3	**Symmetric stent expansion** Defined as minimum lumen diameter divided by maximum lumen diameter $\geq$0.7

MSA minimum stent area

the first study, followed by a sequence of many others later that established IVUS criteria for optimal stent implantation. According to the proposed MUSIC criteria, excellent expansion is evident when the minimum lumen area in the stent is >90 % of the average reference lumen area (Table 2). All the proposed criteria for IVUS optimization used in different studies have relied on distal reference or on mean reference vessel for stent or postdilatation balloon sizing. However, this fact reduces the potential to optimally increase the lumen size particularly in long lesions with overlapping stents and in vessels with distal tapering (de Jaegere et al. 1998; Fitzgerald et al. 2000; Oemrawsingh et al. 2003; Russo et al. 2009).

A large meta-analysis of randomized trials compared IVUS versus angiographic guided BMS implantation and showed that IVUS guidance was associated with significantly lower rate of angiographic restenosis, repeat revascularization, and overall major adverse cardiac events (MACE) but had no significant effect on myocardial infarction (MI) (Parise et al. 2011).

Stent implantation in drug eluting stent (DES) era is associated with very few clinical events. However, the issue of adequate stent implantation becomes even more important with DES, especially in regard to complex, multivessel, and/or left main coronary artery stenting. IVUS predictors associated with PCI failures and increased adverse outcomes with DES include stent underexpansion, edge-related problems like residual reference disease (geographic miss) and dissections, as well

as acute and especially late incomplete stent apposition (malapposition) (Choi et al. 2011, 2012; Fujii et al. 2005; Claessen et al. 2011).

In patients with complex lesions (i.e., bifurcations, long lesions, chronic total occlusions, or small vessels) treated exclusively with DES the use of IVUS demonstrated a benefit in minimum lumen area after stenting comparing to angiography alone. However, no statistically significant difference was found in MACE up to 24 months. In the above randomized AVIO trial the newly proposed criteria for optimal stent expansion was based on the optimal balloon size that should be used for postdilatation. An important attribute of the AVIO criteria is that they can be useful in long lesions, as the stent is evaluated at different segments throughout its length. In addition, these criteria take advantage of the larger vessel size due to positive remodeling (Chieffo et al. 2013).

Whether IVUS guidance reduces stent thrombosis (ST) and improves clinical outcomes associated with DES treatment considered to be controversial. Latest data suggest that IVUS-guided PCI reduce stent thrombosis and improve long-term mortality when compared with angiography-guided PCI after DES implantation. In a very recently published meta-analysis of 11 clinical studies, IVUS-guided DES implantation as compared with angiography guidance alone was associated with a reduced incidence of death, MACE, and stent thrombosis (Zhang et al. 2012).

Likewise, ADAPT-DES was a prospective, multicenter, real-world study of 8583 consecutive patients at 11 international centers undergoing DES implantation to determine the frequency, timing, and its correlation of early and late stent thrombosis. During the index procedure, IVUS was used in 3349 patients. IVUS use resulted in longer stent length and larger stent size without increasing periprocedural MI. This data drawn from the largest prospective registry of IVUS use to date suggests that IVUS guidance during DES PCI may result in less stent thrombosis beginning at the time of implantation as well as fewer myocardial infarctions (Witzenbichler et al. 2014).

Left main coronary arterial lesions are proven to be notoriously difficult to be accurately evaluated by angiography alone. Angiographic appraisal of left main disease correlates very poorly with IVUS and fractional flow reserve (FFR) determinations of lesion severity. This is related to high intra- and interobserver variability as well as the angiographic underestimation of left main dimensions. Moreover, the extent of left main bifurcation plaque burden by IVUS influences PCI outcome, and in general PCI of distal left main bifurcation lesions is related in general with poorer prognosis. IVUS is very useful in distinguishing significant from insignificant left main disease, the distribution of plaque, and planning the appropriate treatment strategy (Oviedo et al. 2010).

By applying predefined IVUS criteria for the assessment of intermediate left main lesions De La Torre et al. showed that an IVUS-derived cutoff of 6 mm^2 can safely determine which intermediate left main lesions require revascularization (de la Torre Hernandez et al. 2011).

In the MAIN-COMPARE registry 975 patients underwent unprotected left main stenting; of those 756 had IVUS guidance and 219 did not. In particular, the comparison between 145 equivalent matched groups of patients who received

Table 3 Basic IVUS measurements

Parameter	Definition – calculation
Vessel diameter	The maximum diameter of the vessel
Lumen diameter	The maximum diameter of the lumen
Vessel area	The circle around the external elastic membrane
Lumen area	The circle around the lumen interface
Stent area	The circle around the stent struts
Plaque area	Vessel area – lumen area
Plaque burden	Vessel area – lumen area / vessel area × 100
Stenosis site	The site with the minimum lumen area
Proximal reference	All the above measurements within 5 mm proximal to a stenosis
Distal reference	All the above measurements within 5 mm distal to a stenosis
Positive remodeling	The enlargement of vessel area compared to proximal reference
Eccentricity	Maximum plaque diameter – minimum plaque diameter at the same frame

DES showed that IVUS guidance in left main PCI was associated with reduced long-term MI and mortality. According to the same data the optimal minimum stent area (MSA) in left main lesions to prevent target lesion revascularization (TLR) was 8.7 mm^2 (Park et al. 2009).

Lately, IVUS has been shown to be an adjunctive imaging technique for the crossing of coronary chronic total occlusions (CTO), the performance of complex aortic, carotid, and peripheral artery endovascular procedures without excluding even vein intervention (Rathore et al. 2010).

Different anatomical criteria should be used according to myocardial mass and/or anatomical variation of coronary artery. As minimum lumen area (MLA) by IVUS has a high negative predictive value, it can be used to exclude the presence of ischemia. Recently, an IVUS-derived MLA $\geq$ 2.4 mm^2 was proposed to exclude functionally significant disease, but below that cutoff, poor specificity limits its value for physiological assessment of lesions (Kang et al. 2011). This is due to the fact that MLA is vessel size dependent and better correlated in large-diameter vessels. The optimal MLA cutoff varies with regard to vessel location, vessel size, and lesion severity. Patients post MI and with reduced LV have higher cutoff MLA. Still, there is no single IVUS or optical coherence tomography (OCT) widely accepted criterion which can be used instead of physiological lesion assessment. All basic IVUS measurements are shown in Table 3 (Mintz et al. 2001). An example of IVUS assessment of an intermediate lesion in a current cath lab setting is given in Fig. 1.

Radiofrequency IVUS Data Analysis (VH-IVUS)

VH-IVUS is an imaging modality that allows tissue characterization of vascular lesions. It is based upon the spectral analysis of the primary raw backscattered ultrasound wave (radiofrequency-based – RF-based – signal). Depending on the

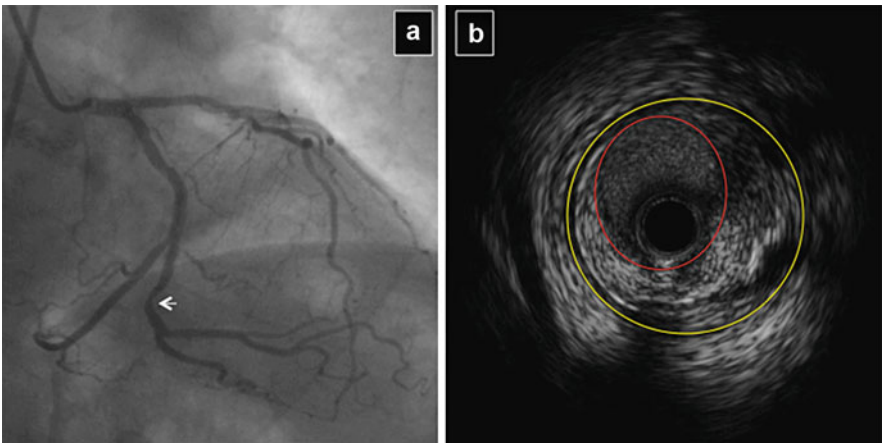

Fig. 1 Intravascular ultrasound assessment in the cath lab. An intermediate angiographic lesion located at the distal LCX (**a**). The minimum lumen area (MLA – *red circle*) measured by IVUS was 4.0 mm^2 and the plaque burden (*yellow circle*) 63 % (**b**)

frequency of the used IVUS catheter the technique has an estimated axial resolution (based on the resolution of the 20 MHz IVUS catheter) of approximately 200 μm. Once the spectral signatures of four tissue types (fibrous tissue, fibrofatty tissue, necrotic core, and dense calcium) are determined, these signatures are programmed into software, either on the IVUS console or stand-alone software packages, for the analysis of patient data. Radiofrequency IVUS plaque components are color coded as dense calcium (white), necrotic core (red), fibrofatty (light green), and fibrous tissue (dark green) (Nair et al. 2007). Technical characteristics and detection capability of IVUS and VH-IVUS are mentioned in Table 4. A case of grayscale and VH-IVUS imaging correlation is shown in Fig. 2.

Ex vivo validation of VH images directly with the histopathology sections provided accuracies of up to 97 % (Garcia-Garcia et al. 2009; Nair et al. 2007). Independent studies have demonstrated in vivo a relatively high level of accuracy and reproducibility of VH-IVUS in human arteries utilizing directional coronary atherectomy specimens yielding predictive accuracies of up to 95 % in non-ACS patients (Nasu et al. 2006, 2008).

The PROSPECT trial tried to assess the natural history of atherosclerosis by studying 697 ACS patients after successful PCI of a culprit lesion under optimal medical therapy using angiography plus three-vessel imaging including grayscale and radiofrequency VH-IVUS. In ACS patients, both culprit and nonculprit lesions were equally likely to spur subsequent adverse events such as cardiac death, cardiac arrest, MI, or rehospitalization due to unstable or progressive angina over 3 years. Independent predictors of a future cardiovascular event were plaques classified as VH-TCFAs (fibroatheroma without evidence of a fibrous cap: >10 % confluent NC with $>30°$ NC abutting the lumen in at least 3 consecutive frames) with a plaque burden >70 % and a minimum lumen area <4 mm^2 (Stone et al. 2011).

Table 4 Technical characteristics and detection capability of IVUS and VH-IVUS

	IVUS	VH-IVUS
Technical characteristics		
Frequency (MHz)	20–45	20–45
Frame rate	10–30	10–30
Pullback speed (mm/s)	0.5–1	0.5–1
Axial resolution (μm)	70–200	70–200
Tissue penetration (mm)	>5	>5
Ease of use	+++	++
Need for contrast	No	No
Detection capability		
Lipid/necrotic core	+	++
Fibrous cap	+	+++
Thrombus	+	No
Calcium	+++	+++
Plaque rupture	++	No
Attenuated plaque	+++	No
TCFA (thin cap fibroatheroma)	No	++
Dissection	++	No
Stent expansion/apposition	++	No
Stent strut coverage	+	+

NA Not applicable
Low capability (+), moderate capability (++), high capability (+++)

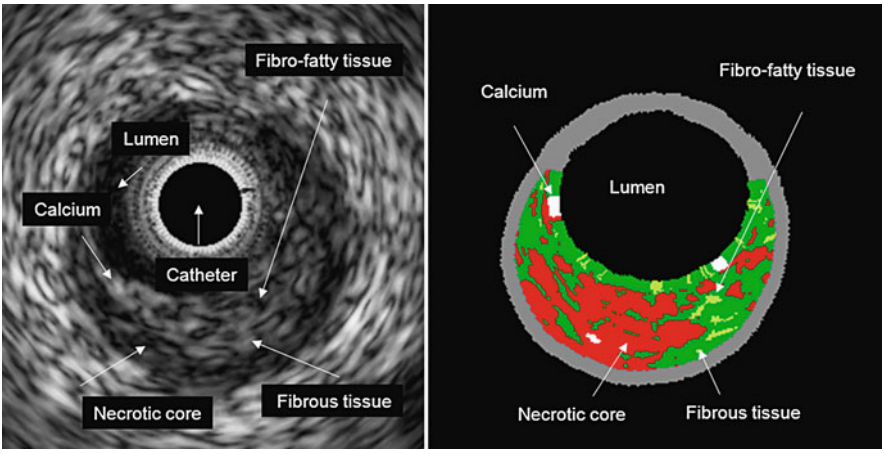

Fig. 2 Gray scale and VH-IVUS imaging correlation. These two cross sectional frames depict the same arterial location and allow visualization of a significant eccentric atherosclerotic plaque. Gray scale intravascular ultrasound (IVUS, *left*) can easily identify lumen and plaque borders but virtual histology VH-IVUS (*right*) provides additional information regarding the compositional plaque characteristics

Similarly, the VIVA study was a prospective analysis of 170 patients with stable angina or ACS who underwent three-vessel VH-IVUS before and after PCI. At a median 1.7 years, 19 lesions (13 nonculprit and 6 culprit) resulted in MACE (death, MI, unplanned revascularization). Nonculprit lesion factors associated with nonrestenotic MACE were VH-IVUS thin-capped fibroatheroma (TCFA) and plaque burden > 70 % TCFA, plaque burden > 70 %, and minimum lumen area < 4 mm^2 were linked with total MACE, suggesting that VH-IVUS can identify plaques at increased risk of subsequent events (Calvert et al. 2011).

Other VH-IVUS data suggested that coronary atherosclerotic plaques with thrombus have very similar compositional characteristics as assessed with grayscale and especially VH-IVUS regardless of whether the angioscopic images showed plaque rupture or absence of plaque rupture. Similarity of VH-IVUS plaque composition (percent NC and percent VH-TCFA) in lesions with or without plaque rupture implies a spectrum of underlying morphologies to explain thrombosis in the absence of a ruptured plaque including classic erosions, small (and undetectable) plaque ruptures, and potentially unruptured TCFAs with superimposed thrombosis (Sanidas et al. 2011).

Controversies exist regarding the association between plaque composition and distal embolization phenomenon after PCI. A large meta-analysis including 16 studies of 1697 patients using IVUS and VH-IVUS data showed that the plaque volume and the necrotic core are closely related to this phenomenon (Jang et al. 2013).

Human coronary atherosclerosis is a dynamic process with potential for replacement of fibrous tissue by necrotic core. VH-IVUS proved to be beneficial in assessing these intraplaque compositional changes and the outcome of pharmacological treatment. The results of the multicenter IBIS-2 trial showed that prolonged pharmacological inhibition halted this process by stabilizing the increase of necrotic core comparing to the placebo group, indicating a direct effect on human atheroma. Regarding the course of coronary plaque regression by statin therapy, another VH-IVUS analysis showed that plaques began to reduce the volume of fibrofatty and fibrous components in the early phase, associated with a transiently increased necrotic core component. Furthermore, even in the case of plaque progression, statins caused a reduction in the necrotic core. However, statin therapy did not halt the incidence in plaque vulnerability (Serruys et al. 2008; Taguchi et al. 2013; Nozue et al. 2013).

VH-IVUS may be also useful in the assessment of complex lesions. A comparison of the distribution of necrotic core in coronary bifurcations showed that bifurcation lesions appear to have a larger plaque burden with a more vulnerable plaque composition compared to nonbifurcation lesions (Garcia-Garcia et al. 2010).

However, other recent data in small cohorts demonstrate that physiological lesion assessment with fractional flow reserve (FFR) do not correlate beyond plaque burden to plaque composition or lesion phenotype as assessed by VH-IVUS (Brugaletta et al. 2012).

Despite the obvious advantages of IVUS-VH regarding the structure of the atheromatous plaque and its potential correlation to clinical end points, there are certain limitations. First, although IVUS-VH can discriminate between some of the

less echogenic components of the plaque (e.g., necrotic core and fibrofatty tissue), separating other soft plaque components, including thrombus, is not currently possible. Moreover, shadowing caused by dense calcific areas can adversely affect correct identification of plaque components. Second, with regard to TCFA identification, it is of note that IVUS-VH axial resolution does not exceed 150 μm and its spatial resolution is even lower (240 μm), while the histological definition of TCFA includes a fibrous cap of 65 μm or less, which means that IVUS-VH cannot accurately assess the thickness of the vulnerable fibroatheroma fibrous cap. Third, IVUS-VH cannot identify cellular components of the vulnerable plaque, such as T-cells and macrophages (Hartmann et al. 2009; Sawada et al. 2008).

In conclusion, although VH-IVUS is an excellent research tool there is currently no robust data supporting its routine use in PCI.

Future Directions

To date, several different modalities have been proposed regarding the three-dimensional (3D) reconstruction of the coronary arteries integrating angiographic and IVUS data. These methods are mainly based on the fusion of data obtained by biplane angiography and IVUS using a segmentation algorithm for the detection of the regions of interest in IVUS images and a new methodology for the extraction of the catheter path from angiographic images. All of them can provide rapid coronary reconstruction allowing accurate estimation of lesion dimensions and determination of plaque distribution and volume (Gogas et al. 2013; Bourantas et al. 2008; Teeuwen et al. 2011).

Recently, another RF-based processing method has been presented for in vivo coronary plaque tissue characterization: the i-MAP-IVUS (Boston Scientific, Santa Clara, California). From a methodological point of view this software is comparable to the VH-IVUS system; however, there are differences, such as the applied color scheme: (1) Fibrous tissue (light green), (2) Lipid tissue (yellow), (3) Necrotic core (pink), and (4) Calcium (blue). Furthermore, the applied IVUS catheter is the 40 MHz rotating single-element catheter instead of the 20 MHz mechanical one used with VH-IVUS. Ex vivo validation demonstrated accuracies at the highest level of confidence as 97 %, 98 %, 95 %, and 98 % for necrotic, lipid, fibrotic, and calcified regions respectively (Sathyanarayana et al. 2009; Garcia-Garcia et al. 2011).

Beyond the classic VH analysis that provides qualitative and quantitative information of the different plaque components in terms of their percentage and area (mm^2) within the plaque region, a novel approach has been also proposed. By post hoc analysis of VH-IVUS images, the computational quantification of new structural features of coronary plaques has been shown to provide new compositional and structural indices which indicate spatial distribution, heterogeneity, and dispersity of each VH-IVUS-derived component within the plaque area and also with respect to the plaque-lumen border (Papaioannou et al. 2014).

Elastography is another IVUS-based method that has been used to assess the deformation of plaques through the changes in intracoronary pressure that occur during the cardiac cycle reflecting the mechanical properties of the vessel wall. This technique can characterize the softness of plaques, which might be a sign of vulnerability prior to rupture (Schaar et al. 2003).

Of interest, IVUS has also been used to study shear stress produced by coronary artery blood flow, which may explain the localization of early plaque, TCFAs, and culprit lesions. The technique uses 3D images of the vessel and computational fluid dynamics to calculate the force directed along the endothelial surface of the vessel wall resulting from the friction associated with blood flow. Plaque formation is more likely to originate at sites that have lower shear stress which predisposes to inflammation and endothelial dysfunction (Malek et al. 1999). It has been also found that coronary artery wall shear stress is associated with the progression and transformation of atherosclerotic plaque and arterial remodeling in a prospective study of 20 patients with coronary artery disease (Samady et al. 2011). Also the combination of plaque burden, wall shear stress, and plaque phenotype – as assessed by VH-IVUS and 3-D artery geometry and blood flow profile – has incremental value for prediction of coronary atherosclerotic plaque progression and vulnerability (Corban et al. 2014).

Contrast-enhanced IVUS imaging (CE-IVUS) is a novel, yet clinically available, technique that has the potential to enhance IVUS-based in vivo characterization of atherosclerotic plaques by detecting the density of vasa vasorum and the neovasculature that nourish the plaque and the vessel wall. Recent evidence has suggested that the presence and proliferation of vasa vasorum neoangiogenesis in the plaque can be correlated to an increased inflammation process leading to plaque vulnerability. Based on this evidence, IVUS is used in combination with contrast agents (microbubbles) for the qualification and quantification of extraluminal blood perfusion which might be an indication of vasa vasorum. The method is supported by a custom-made software (Vavuranakis et al. 2007, 2008).

Chemically specific optical absorption spectra can be used for tissue identification in ultrasonic imaging of atherosclerosis. Recent experimental developments using a combined IVUS / photoacoustics imaging system indicate that sound and light is the way to go for the diagnosis of vulnerable plaque. This hybrid imaging technique combines the advantages of high spatial resolution of ultrasound with contrast of optical absorption. Photoacoustic imaging can distinguish the major lipid components of atherosclerotic plaques and also differentiate between lipids present in atherosclerotic plaques from lipids present in periadventitial tissue (Wang et al. 2012; Bourantas et al. 2013).

Additional efforts may include the development of a magnetic resonance catheter-based system that can identify lipid-rich tissue or even imaging catheters able to measure thermal gradients associated with inflammation in the coronary arteries. Finally, molecular imaging agents may enhance identification of specific molecular processes within the plaques (Stefanadis et al. 1999; Wilensky et al. 2006; Jaffer and Weissleder 2005).

Limitations

Despite the profound advantages of IVUS in the assessment of atherosclerosis in vivo their major limitation is mainly related to the fact that it is invasive. In order to provide their unique information it is mandatory to be held in a cath lab setting under experienced operators and staff. Although the rate of procedural complications remains low (1 %) it still exists. It includes a wide variety of related pitfalls including mainly vasospasm and less often dissections, perforations, and induced arrhythmias. Prolonged radiation exposure and increased contrast usage should be also taken under consideration. From a technical point of view the need to catheterize each vessel individually is also a matter of time and concern and relies always on the experience and skills of the interventional cardiologist. Anatomically speaking another restriction is related to their limited capability of imaging small-diameter vessels and aorto-ostial lesions. In addition, as with any visualization modality, certain artifacts may occur such as the ring down, geometric distortion effect, blood speckle, nonuniform rotational distortion, or even broken catheters and devices. Another major concern is that image analysis should be always performed by experts with obtained training in the field otherwise it might lead to inaccurate and misleading interpretation and in a not-favorable outcome. Last but not least the high cost of these machines and catheters and the occasionally limited availability of each product due to approval or distribution issues remains a restriction to their worldwide spread.

Potential Applications to Prognosis, Other Diseases or Conditions

Among other applications, IVUS has evolved a niche role in assessing the proper deployment of stents at the conclusion of an endovascular intervention. The role of imaging modalities in the management of peripheral arterial disease (PAD) is crucial, with conventional digital subtraction angiography (DSA) considered the gold standard tool for the diagnostic assessment and endovascular treatment of PAD. IVUS has the ability to overcome several pitfalls of DSA. IVUS can image vessels in a cross-sectional plane and provide information regarding the morphology of the lesion and the vessel wall, precise cross-sectional measurements, and the location of important branch vessels. It also provides spatial relationships between a deployed stent and the vessel wall, including the adequacy of stent apposition.

Another recent advancement contributing to the accuracy of diagnosis of renovascular disease, namely renal stenosis and fibromuscular dysplasia (FMD), is the use of IVUS. IVUS gives a detailed understanding of the severity of the stenosis and assists in accurate sizing of balloons. Also, utilizing IVUS to evaluate restenosis after endovascular therapy for FMD can be extremely valuable.

The use of IVUS has also been expanded in endovascular venous procedures in order to identify the mechanisms of acute vein closure as well as incomplete stent apposition after intervention. It has been used on femoral, internal carotid,

subclavian, brachiocephalic, polpiteal veins and/or even vein grafts after bypass surgery. A potential limitation is that the absence of adjacent artery or anatomical landmarks is often not available or can be misleading. Nonetheless, IVUS and venography provide complementary data for diagnosis, sizing, results, and complications.

Finally, recent specialized modifications, such as novel hybrid and integrated built-up systems, enhanced image resolution software, the use of 3-D reconstructed images, the analysis of radiofrequency backscatter data, the use of automated detection techniques, and the development of this knowledge, may all help expanding its clinical use.

Summary Points

- This chapter focuses on the interpretation of coronary artery disease with intravascular ultrasound (IVUS).
- Coronary angiography often underestimates the degree of intraluminal stenosis and does not gauge the size of the plaque itself.
- IVUS provides real-time, high-resolution images of the coronary arteries and unique insights of atherosclerotic plaque morphology such as size, eccentricity, and calcification.
- IVUS can be also used for a detailed evaluation of stent implantation by assessing lesion morphology, vessel size, lumen dimensions, apposition, and plaque distribution before and after stenting.
- Processing the IVUS radiofrequency signal using the VH-IVUS software offers a more precise tissue characterization of the composition of atherosclerotic plaques.

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Markers and Correlates of Right Ventricular Function with Computed Tomography, Echocardiography, and Magnetic Resonance

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_39

Abstract

Right ventricle (RV) dysfunction has important prognostic value in many cardiovascular conditions. Unfortunately, the assessment of this ventricle with current imaging modalities is challenging, but some biomarkers show promises to supplement this difficult evaluation. This chapter reviews the relationship of the most common biomarkers studied in association with RV imaging techniques.

The natriuretic peptides (NP) BNP and NT-proBNP have good diagnostic accuracy for RV dilation and dysfunction in the settings of acute pulmonary embolism, chronic pulmonary hypertension, and arrhythmogenic RV cardiomyopathy. In adult with congenital heart diseases, the NP seem to correlate with RV function, but great variability exists depending on the type of cardiac defects studied. sST2 has recently gained attention as a potentially interesting biomarker, but its use in right-sided diseases remains scarce and experimental. In acute decompensated heart failure (HF), it relates only modestly with some of the echocardiographic parameters of RV function. Results are more encouraging in pulmonary hypertension, with significant correlations between sST2 and RV size and function. Cystatin C is a well-recognized marker of kidney dysfunction and seems to have some correlation with RV function in patients with HF and pulmonary hypertension. NGAL and galectin-3 are among the latest biomarkers studied in cardiology, but their relation with the RV imaging is unclear at the present time. Finally, evidences are somewhat conflicting for RV evaluation; while their levels do not correlate with RV size and function in acute pulmonary embolism, they are predictors of mortality.

In conclusion, RV dysfunction encompasses a very heterogeneous group of patients with various pathologies and prognosis; the use of biomarkers must therefore be adapted to these multiple clinical situations. Unfortunately, there is no universal biomarker for the evaluation of the RV but some show promises.

Keywords

Biomarker • BNP • Cystatin C • Galectin-3 • NGAL • NT-proBNP • sST2 • Troponin • RV

Abbreviations

ARVC	Arrhythmogenic right ventricular cardiomyopathy
ASD	Atrial septal defect
AUC	Area under curve
BMI	Body mass index
BNP	B-type natriuretic peptide
CMR	Cardiac magnetic resonance
COPD	Chronic obstructive pulmonary disease
CT	Computed tomography

eGFR	Estimated glomerular filtration rate
hsTnI	High-sensitivity troponin I
hsTnT	High-sensitivity troponin T
IQR	Interquartile range
LV	Left ventricle
LVAD	Left ventricular assist device
LVEF	Left ventricle ejection fraction
MPI	Myocardial performance index
NGAL	Neutrophil gelatinase-associated lipocalin
NT-proBNP	N-terminal prohormone brain natriuretic peptide
PAP	Pulmonary arterial pressure
PWD	Pulsed wave Doppler
RV	Right ventricle
RVEDD	Right ventricle end-diastolic diameter
RVEDV	Right ventricle end-diastolic volume
RVEF	Right ventricle ejection fraction
RVESV	Right ventricle end-systolic volume
RVFAC	Right ventricular fractional area change
RVSP	Right ventricle systolic pressure
TAPSE	Tricuspid annular plane systolic excursion
TDI	Tissue Doppler imaging
TR	Tricuspid regurgitation

Key Facts of ...

Key Facts of the Right Ventricle

- The right ventricle (RV) is the cardiac chamber normally receiving the venous blood from the peripheral venous circulation via the right atrium. It pushes this deoxygenated blood in the pulmonary circulation through the pulmonary artery. The lungs will then proceed to vital gas exchanges and reoxygenation of the blood before returning it to the left heart (left atrium and left ventricle).
- The RV is different from the left ventricle in many ways, including:
  - Its wall is composed of only one muscle layer (three for the left ventricle) and is thinner (<5 mm).
  - It has a crescent shape wrapped around the left ventricle.
  - Its pressure-volume loop is triangular with very short isovolumic contraction and relaxation periods. An increase in its afterload will reduce its performance much more than it would for the left ventricle.
  - It is difficult to image the RV and it is subject to higher variability than the left ventricle, which has more of a cone shape.
  - The normal RV ejection fraction (RVEF) is lower than the left ventricle ejection fraction; >45 % (measured by cardiac magnetic resonance) compared to ≥ 55–60 % for the left ventricle.

Key Facts of Echocardiogram

- Echocardiogram is a test using ultrasounds to image the heart and measure noninvasively hemodynamic variables.
- It is easily available and safe. However, poor acoustic windows can preclude adequate evaluation in some patients.
- Doppler echocardiography uses the changes in ultrasound reflected by red blood cells to derive the velocity of the blood. Physics and mathematics principles can then be applied to derive pressure gradients and obtain an extensive hemodynamic cardiac evaluation.
- Tissue Doppler imaging uses the velocities of the myocardial tissue. It can be used to derive markers of the systolic and diastolic function of both ventricles.
- Strain imaging uses tissue deformation velocity during the cardiac cycle to estimate ventricular function. Speed of the shortening between two points in the ventricle is proportional to the wall stress.

Key Facts of Cardiac Magnetic Resonance

- In cardiac magnetic resonance, image acquisition is gated to an electrocardiogram. It allows an excellent assessment of both ventricle function and anatomic description.
- Cardiac magnetic resonance is currently the gold standard for RV volumes and ejection fraction assessment.
- Cardiac tissue characterization is possible using intravenous gadolinium and different sequences/settings. This allows ventricular edema and fibrosis visualization and quantification.
- Availability of this technique can be problematic: cost is higher than other cardiac imaging modalities and it is not available in most non-tertiary healthcare centers.
- Cardiac magnetic resonance is contraindicated in patients with pacemaker or defibrillator. Other metallic implants can also be a contraindication.

Key Facts of Cardiac Computed Tomography

- Computed tomography uses X-rays to obtain cross-sectional images of the body. Intravenous contrast agent can be used to enhance differentiation between tissues.
- When gated to an electrocardiogram, it can be used to image the heart and obtain volumes and ejection fraction.
- Its use is limited by the risk of nephrotoxicity caused by contrast agent and the long-term cancer risk associated with radiation exposure.
- Specific settings of the computed tomography scanner and software are necessary to obtain accurate cardiac imaging. This is not currently available in all healthcare centers.

Definitions

Arrhythmogenic right ventricular cardiomyopathy This is a genetic condition characterized by fibro-fatty replacement of the myocardium, affecting predominantly the RV. It is associated with lethal ventricular arrhythmias but also with progressive heart failure.

Cardiorenal syndrome Renal dysfunction observed in the setting of heart failure is complex and called cardiorenal syndrome. Poor kidney perfusion, systemic venous congestion, and neurohormonal activation secondary to heart failure are all contributors. Both organs can perpetuate and worsen the function of the other one.

Congenital heart disease This term encompasses a wide range of cardiac malformations that happened during embryogenesis. The type and severity of the defects will dictate the treatment, most often surgical.

Ejection fraction An estimate of the amount of the blood ejected by the ventricle each systole compared to the maximal volume of the blood contained by the ventricle before ejection at end-diastole. It is expressed as a fraction or a %. It can be obtained by echocardiogram, cardiac magnetic resonance, cardiac computed tomography, or nuclear medicine imaging.

Myocardial performance index It is an echocardiographic measure of the isovolumic contraction and relaxation time versus systolic ejection time. It provides an estimate of the ventricle performance. Normal values for the RV are ≤ 0.4 by pulsed wave Doppler and ≤ 0.55 by tissue Doppler. It is also called Tei index or MPI.

Pulmonary hypertension Higher than the normal arterial blood pressure in the lung circulation; is defined as mean pulmonary arterial pressure ≥ 25 mmHg. Most importantly for diagnostic and treatment, pulmonary *arterial* hypertension (caused by primary disease of the pulmonary arterial bed) should be differentiated from post-capillary pulmonary hypertension (caused by high left heart-filling pressures).

Tricuspid annular plane systolic excursion The tricuspid annular plane displacement from diastole to systole is measured using echocardiographic tissue Doppler imaging. It provides an estimate of the RV performance. The normal value is ≥ 16 mm.

Introduction

The presence of right ventricular (RV) dysfunction has important prognostic value in many cardiovascular conditions. Unfortunately, the assessment of RV size and function is challenging using available imaging modalities. Nuclear medicine, computed tomography (CT), and echocardiography are commonly used but they

all have significant limitations. Cardiac magnetic resonance (CMR) is the gold standard for RV evaluation but has limited availability and is often contraindicated. On the other hand, the use of biomarkers in cardiology has become an essential tool for clinical decision-making in many situations. In heart failure (HF) patients, biomarkers are used for their diagnostic and prognostic values and to guide treatment. While most of the literature relates to heart failure with reduced left ventricle (LV) ejection fraction, some biomarkers have shown promises for right-sided diseases and may have a role complementary to RV imaging. In this chapter, the relationships between imaging modalities and some of the currently available biomarkers will be reviewed for pathologies commonly affecting the RV.

Cystatin C

Cystatin C is a cysteine protease inhibitor produced and released by all nucleated cells. It has a low molecular weight and is freely filtered by the glomeruli to then be absorbed and catabolized in the proximal tubule. Its level is related to cellular turnover but mainly to glomerular filtration rate (GFR); hence it has been extensively studied as a marker of kidney function. It seems to perform better than creatinine due to the absence of variation with age, gender, and muscle mass (Lassus and Harjola 2012).

Heart Failure and Systolic Dysfunction

Cystatin C level has been shown to be a powerful biomarker in HF, where it can predict long-term mortality, HF hospitalization, and cardiac transplantation in both acute and chronic situations (Tang et al. 2008; Lassus and Harjola 2012). High level shows significant correlation with NT-proBNP level but also with LV diastolic dysfunction and mitral regurgitation severity by echocardiography (Tang et al. 2008). It seems to be directly involved in the cardiac remodeling process, with higher level of cystatin C found in the serum and cardiac tissue of mice with doxorubicin-induced cardiomyopathy or after myocardial infarction (Xie et al. 2010) and also with LV hypertrophy in patients without HF (Ix et al. 2006).

In 139 stable patients with HF and LV ejection fraction (LVEF) $\leq$35 %, cystatin C exhibited a modest but significant correlation with RV dysfunction visually assessed by echocardiography (median cystatin C level:1.22; IQR:1.03–1.62 mg/L; and $r = 0.30$, $p < 0.001$). Increasing quartiles showed greater likelihood of at least moderate RV systolic dysfunction: OR = 4.58, IQR:1.26–21.98 for third quartile and OR = 5.91, IQR:1.67–28.08 for fourth quartiles (all $p < 0.05$) (Tang et al. 2008). Interestingly, in the same study, cystatin C level showed correlation with mitral regurgitation severity, mitral E/E' ratio, but neither with LVEF nor indexed LV end-diastolic volume (LVEDV). Similar correlation between RV end-diastolic diameter (RVEDD) by echocardiogram and cystatin C was likewise demonstrated in patients with stable dilated cardiomyopathy and LVEF $\leq$40 % ($r = 0.38$, $p = 0.01$) (Bielecka-Dabrowa et al. 2013). In that group LVEF and LV

Table 1 Cystatin C levels in different pathologies affecting the RV and correlations with imaging

Pathology	First author, publication year	Nb of patients, mean eGFR, mean RV function, and size	Patients Cystatin C, median (IQR), or mean ± SD	Nb of controls and definition	Controls Cystatin C, median (IQR), or mean ± SD	Cystatin C correlations with RV imaging
Systolic heart failure	Bielecka-Dabrowa et al. (2013)	45 patients with dilated CMP (LVEF ≤40 %), eGFR: 77.23 ± 22, RVEDD: 2.98 ± 0.5 MPI PWD: 0.69 ± 0.30	1.1 ± 0.33 mg/L	–	–	Echo: RVEDD $r = 0.38$, $p = 0.01$
	Pronschinske et al. (2014)	40 patients with VAD implanted and eGFR 53.0 ± 33	VAD implanted and RV failure post-op: 1135.4 ± 231.3 ng/mL	40 patients with stable HF (NYHA II-III), eGFR : 66.0 ± 22 24 healthy controls, eGFR 76.5 ± 17	VAD implanted but no RV failure post-op: 1016.9 ± 580.8 ng/mL (p NS vs. RV failure) Stable HF: 1490.4 ± 576.1 ng/mL Controls: 954.7 ± 414.2 ng/mL	–
	Tang et al. (2008)	139 patients with chronic stable HF (LVEF ≤35 %); 95/139 had eGFR ≥60 25 % had ≥ moderate RV systolic dysfunction	1.22 (1.03–1.62) mg/L	–	–	Echo: semiquantitative visual estimate of RV systolic function: $r = 0.30$, $p < 0.001$

(*continued*)

Table 1 (continued)

Pathology	First author, publication year	Nb of patients, mean eGFR, mean RV function, and size	Patients Cystatin C, median (IQR), or mean ± SD	Nb of controls and definition	Controls Cystatin C, median (IQR), or mean ± SD	Cystatin C correlations with RV imaging
Pulmonary arterial hypertension	Fenster et al. (2014)	14 patients with PAH, eGFR 71 (48–101) RVEF: 35 % (27–39) TAPSE: 17 (16–19) RV MPI PWD: 0.41 (0.26–0.53) RVSP: 57 (46–65)	1.00 ± 0.23 mg/L	10 healthy controls, eGFR 75 (70–84)	0.78 ± 0.05 mg/L	CMR: RVEF: $r = -0.58$, $p = 0.003$ RVMi: $r = 0.66$, $p = 0.0004$ RVEDV: $r = 0.50$, $p = 0.01$ RVESV: $r = 0.58$, $p = 0.003$ Echo: TAPSE: $r = -0.45$, $p = 0.03$ RV lat. peak strain: $r = 0.51$, $p = 0.01$ MPI PWD: NS RVSP: $r = 0.61$, $p = 0.002$ TV E/e': $r = 0.75$, $p \leq 0.0001$ TV E/e': $r = 0.57$, $p = 0.003$ TV E/A: $r = -0.50$, $p = 0.01$

This table summarizes the published studies on the relationship between cystatin C and function and/or size of the RV on imaging. They are separated between pathologies affecting the RV

Abbreviation key: *CMP* cardiomyopathy, *CMR* cardiac magnetic resonance, *echo* echocardiogram, *eGFR* estimated glomerular filtration rate (cc/min/1.73 m^2), *HF* heart failure, *IQR* interquartile range, *LVEF* left ventricle ejection fraction, *MPI* myocardial performance index, *Nb* number, *NS* nonsignificant, *PAH* pulmonary arterial hypertension, *PWD* pulsed wave Doppler, *RV* right ventricle, *RVEDD* right ventricle end-diastolic diameter (mm), *RVEDV* right ventricle end-diastolic volume (mL), *RVEF* right ventricle ejection fraction, *RVESV* right ventricle end-systolic volume (mL), *RVMi* right ventricle mass indexed (g/m^2), *RVSP* right ventricle systolic pressure (mmHg), *SD* standard deviation, *TAPSE* tricuspid annular plane systolic excursion (mm), *TV* tricuspid valve, *VAD* ventricular assist device

size had significant correlations with cystatin C. On the other hand, cystatin C was not able to predict clinical RV failure after left ventricular assist device (LVAD) in a group of 40 patients with advanced HF nor did estimated GFR, which contrasts with previous reports (Pronschinske et al. 2014) (Table 1).

The observed relationship between RV parameters and cystatin C level has been proposed to be secondary to RV impairment causing venous congestion leading to cardiorenal syndrome. Exception of the very specific situation of RV failure post LVAD implantation, data are encouraging for cystatin C as a potential marker of RV dysfunction in HF population.

Pulmonary Arterial Hypertension

Fenster et al. performed a multimodal study including CMR, echocardiogram with tissue Doppler imaging (TDI), and speckle-tracking imaging in 14 patients with pulmonary arterial hypertension and normal eGFR compared to 10 matched controls (Fenster et al. 2014). Significant correlations were observed between cystatin C and multiple markers of RV remodeling ($r = 0.5$–0.66), systolic ($r = 0.45$–0.61), and diastolic function ($r = 0.5$–0.75, all $p \leq 0.01$). The only echocardiographic surrogate of RV function not significantly correlated to cystatin C was RV myocardial performance index (RV-MPI) using pulsed wave Doppler (PWD), which incorporates both elements of systolic and diastolic dysfunction; of note this index did not correlated either with natriuretic peptide (NP) levels. These findings suggest a role for cystatin C as a marker of RV function and remodeling, irrespective of the presence of cardiorenal syndrome; nevertheless, further research is needed before incorporating this biomarker in routine clinical practice.

Galectin-3

Galectin-3 is a β-galactoside-binding lectin expressed by active macrophages during phagocytosis. Upregulation of galectin-3 has been linked to active fibrosis in multiple organs and plays a contributory role in cardiac remodeling process by directly influencing matrix components (de Boer et al. 2009), where it can bind to the fibroblast and induce expression of myocardial collagen and interstitial fibrosis (Sharma et al. 2004). In a subgroup of 115 patients with acute HF enrolled in the PRIDE trial (Januzzi et al. 2005; Shah et al. 2010), galectin-3 level was an independent predictor of mortality but did not correlate with LV or left atrium (LA) dimensions or LVEF. However, correlations were present with mitral regurgitation severity ($r = 0.3$) and LV diastolic dysfunction parameters (E' peak velocity $r = -0.25$; E/E' ratio $r = 0.35$; all $p < 0.05$). Low but significant coefficients were also found between galectin-3 and right ventricle fractional area change (RVFAC) ($r = -0.193$), right ventricle systolic pressure (RVSP) ($r = 0.37$), tricuspid regurgitation (TR) jet velocity ($r = 0.304$), and TR severity ($r = 0.26$, all $p \leq 0.05$) in the same study.

Galectin-3 is believed to be a marker of fibrosis; whether correlation with RV function is related to changes in left-sided filling pressures or/and that remodeling and fibrosis observed in LV can also affect the RV remains unknown at the present time. Further research is warranted to elucidate the role of galectin-3 in the evaluation of RV function.

Natriuretic Peptides

B-type natriuretic peptide (BNP) and the inactive N-terminal fragment of its precursor proBNP (NT-proBNP) are the most extensively studied natriuretic peptides (NP). BNP is secreted mainly by the LV in response to wall stress caused either by increased pressure or volume (Nishikimi et al. 2011). This counter-regulation hormone acts through membrane-bound NP receptors to induce vasodilatation, natriuresis, and diuresis (Nakao et al. 1992). NP levels increase with age and female gender (Redfield et al. 2002) and decrease with higher kidney function (Lamb et al. 2006) and body mass index (BMI) (Mehra et al. 2004). Most of the abundant literature on NP relates to HF for diagnostic, prognostic, and monitoring purposes. Interestingly, other cardiac chambers can also secrete BNP, such as the atria and the RV (Hosoda et al. 1991; Matsuo et al. 1998); RV pressure and volume overloads are associated with increased BNP level, even in the presence of normal LV hemodynamics (Nagaya et al. 1998). While accepted cutoff exist for excluding the diagnostic of HF in patients presenting with dyspnea in the emergency department (BNP <100 pg/ml (Maisel et al. 2002) and NT-proBNP <300 pg/ml (Januzzi et al. 2005)), no such value exists for right-sided diseases.

Right Ventricle Function and Left Ventricle Systolic Heart Failure

The contribution of the RV to the NP levels measured in patients with systolic LV dysfunction has not been well studied. In a retrospective analysis of 246 ambulatory HF patients on recommended guidelines-derived medical therapy, right ventricle ejection fraction (RVEF) measured by first-pass radionuclide angiography exhibited a significant relationship with BNP level. The mean RVEF was 38 ± 10 % and median BNP was 158 pg/mL (IQR: 374 pg/mL); every 10 % decrease in RVEF was associated with an increase of logBNP of 0.26 ($p < 0.05$), after adjustment for multiple potential confounding variables (Murninkas et al. 2014). While these findings suggest a direct relationship between RV function and BNP, it is impossible to discriminate elevation explained by RV failure itself in patients with concomitant LV dysfunction.

Pulmonary Embolism

Acute pulmonary embolism (PE) creates pressure overload on the RV that can trigger BNP secretion and the NP carry important prognostic value in this situation.

A massive PE with hypotension is associated with increased mortality usually as a consequence of acute RV failure. Even in normotensive patients with acute PE, elevated NP levels suggest RV dysfunction as seen on echocardiogram and CT pulmonary angiography. In a group of 152 patients with acute PE, BNP level was significantly higher in RV dysfunction group (129, IQR:41–448 pg/ml) compared to patients with RV dilation but normal function (22, IQR:10–78 pg/ml, $p = 0.005$) and those with normal RV size and function (12, IQR:0–63 pg/ml, $p = 0.001$) (Kline et al. 2008). RV involvement by echocardiogram was defined as the presence of a McConnell sign (hypokinesia of the infundibulum with normal RV apical contraction) or markedly depressed RV contractility. BNP cutoff of 100 pg/ml had a fair diagnostic accuracy for detecting RV hypokinesis, with a likelihood ratio of 2.8, area under curve (AUC) of 0.71 (0.62–0.80), sensitivity of 57 %, and specificity of 78 %. Similar findings were obtained using CT pulmonary angiography, where RV dilatation was defined as axial RV/LV diameter ratio >1.0 (Vuilleumier et al. 2008). Median BNP was 170 pg/ml (IQR 68–261 pg/ml) in those with RV dilatation compared to 36 pg/ml (IQR 23–88) in patients with RV/LV ratio <1.0. Correlation was modest but significant ($r = 0.28$, $p = 0.047$), and a BNP cutoff level of 100 pg/ml had similar diagnostic accuracy for RV dilatation by CT (AUC = 0.72, sensitivity = 65 % (45–81 %), and specificity = 78 % (45–81 %)) (Table 2).

Similar correlation was observed between NT-proBNP and RV dilatation by echocardiogram ($r = 0.37$, $p = 0.001$ Ozsu et al. 2010). Further, Henzler and colleagues used a comprehensive echocardiographic evaluation for RV dysfunction requiring two or more of the following criteria: dyskinesia/hypokinesia of RV free wall, positive McConnell sign, tricuspid annular plane systolic excursion (TAPSE) <15 mm, RVSP >30 mmHg, RV diameter >30 mm, or RV/LV end-diastolic diameter ratio >1 (apical 4-chamber view) (Henzler et al. 2012). Patients with RV dysfunction exhibited a significantly higher NT-proBNP than those with normal RV function (6,372 ± 2,319 vs. 1,032 ± 1,559 ng/L, $p = 0.002$). NT-proBNP was a significant predictor of RV dysfunction by multivariate analysis (OR = 5.0, 95 % CI:1.6–15.9, $p = 0.019$), and a NT-proBNP cutoff of 1,617 ng/L had significant diagnostic accuracy for RV dysfunction (AUC = 0.83, sensitivity = 75 %, and specificity = 80 %). Furthermore, a NT-proBNP cutoff of ≥1,840 ng/L suggested severe RV dysfunction, defined as bulging of the interventricular septum into the LV (AUC = 0.93, sensitivity = 100 %, specificity = 81 %), and a cutoff of 1,427 ng/L suggested moderate RV dysfunction (AUC = 0.80, sensitivity = 75 %, specificity = 72 %). Comparable findings were obtained with CT pulmonary angiography, with NT-proBNP correlating with RV/LV diameter ratio ($r = 0.280$–0.68, all $p < 0.05$) (Vuilleumier et al. 2008; Ozsu et al. 2010; Henzler et al. 2012; Laiho et al. 2012). Levels of BNP and NT-proBNP varied greatly in these studies, depending on the assay used and the patient's population; nevertheless, NP are useful adjunct to clinical evaluation and imaging in the characterization of patients with PE for prognostication and identifying patients with potential RV dysfunction.

Table 2 BNP and NT-proBNP levels, correlations with imaging, and diagnostic accuracies for RV dysfunction and/or dilatation in acute pulmonary embolism

First author, publication year	Nb of patients, mean age, nb of men, mean eGFR, mean BMI	RV dysfunction definition, nb of patients	BNP or NT-proBNP median (IQR or range) in RV dysfunction group	BNP or NT-proBNP median (IQR or range) in normal RV group	Cutoff and AUC for RV dysfunction on imaging	Sensitivity and specificity	Other correlations with imaging
BNP							
Kline et al. (2008)	152, 52–56 years old, sex not given, eGRF not given, 29–31	RV hypokinesia (echo) (McConnell sign or qualitative hypokin)	129 (41–448) pg/mL	12 (0–63) pg/mL	100 pg/mL	Sensitivity: 57 % Specificity: 78 %	–
		37/152 patients			AUC: 0.71 (0.62–0.80)		
		5 had LVEF <45 %					
		RV dilatation (echo)	22 (10–78) pg/mL	12 (0–63) pg/mL	–	–	–
		36/152 patients					
		4 had LVEF <45 %					
Vuilleumier et al. (2008)	50, 74 years old, 21 men, median creat 87 umol/L, BMI not given	CTPA RV/LV diameter >1.0	170 (68–261) pg/ml	36 (23–88) pg/ml	100 pg/ml	Sensitivity: 65.2 (45–81)%	RV/LV index: $r = 0.28$, $p = 0.047$
		23/50 patients			AUC: 0.715	Specificity: 77.8 (45–81)%	
NT-proBNP							
Henzler et al. (2012)	77, 63 years old, 42 men, CKD in 4/77, BMI not given 13/77 had CHF	RVD if ≥2 echo criteria: dyskinesia or hypokin of RV free-wall, McConnell sign, TAPSE <15 mm, RV/atrial gradient >30 mmHg, RV diameter >30 mm or RV/LV end-diastolic diameter ratio >1 in apical 4-chambers 27/77 patients	6,372 ± 2,319 ng/L	1,032 ± 1,559 ng/L	1,617 ng/L AUC: 0.83	Sensitivity: 75 % Specificity: 80 %	CTPA RV/LV axial: $r = 0.38$ CTPA RV/LV 4 chambers: $r = 0.52$ CTPA RV/LV volume: $r = 0.68$

Laiho et al. (2012)	63, 55 years old, 30 men, eGFR not given, BMI >25 in 50 patients	CTPA RV/LV diameter >1 and/or interventricular septum deviation	86 % had NT-proBNP >350 ng/L	27 % had NT-proBNP >350 ng/L	–	–	–
	Normal LVEF in all	37/63 patients					
Ozsu et al. (2010)	108, 70 years old, 47 men, eGFR and BMI not given	CTPA RV/LV dimension ≥1.1	129 (5–555) pmol/ml	42 (5–758) pmol/ml	–	–	–
		48/108 patients					
	9 % had comorbid HF	Echo RV end-diastolic diameter >30 mm	173 (5–758) pmol/L	48 (5–539) pmol/L	–	–	–
		44/108 patients					
Vuilleumier et al. (2008)	50, 74 years old, 21 men, median creat 87 umonl/L, BMI not given	RV/LV diameter ratio >1.0 on CTPA	1,369 (165–2,856) pg/ml	170.7 (85–910) pg/ml	300 pg/ml	Sensitivity: 73.9 (54–87)%	RV/LV index: $r = 0.36$, $p = 0.009$
		23/50 patients			AUC: 0.748	Specificity: 66.7 (48–81)%	

This table summarizes the published studies on the relationship between BNP/NT-proBNP and function and/or size of the RV on imaging in patients with acute pulmonary embolism. They are separated between BNP and NT-proBNP for easier comparison

Abbreviation key: *AUC* area under curve, *BMI* body mass index (kg/m^2), *CHF* congestive heart failure, *CKD* chronic kidney disease, *creat* creatinine, *CTPA* computed tomography pulmonary angiogram, *echo* echocardiogram, *eGFR* estimated glomerular filtration rate (cc/min/1.73 m^2), *IQR* interquartile range, *LVEF* left ventricle ejection fraction (%), *nb* number, *RV* right ventricle, *RVD* right ventricle dysfunction, *TAPSE* tricuspid annular plane systolic excursion (mm)

Pulmonary Hypertension

NP are powerful prognostic markers of survival in patients with pulmonary hypertension (PH) and are recommended as an integral part of the evaluation of patients with PH (Galie et al. 2009). NP levels have shown good correlation with different RV-imaging modalities in many clinical situations. In PH secondary to chronic volume overload (atrial septum defect) or pressure overload (primary arterial PH and chronic thromboembolic PH), BNP level had no correlation with RV volumes but was strongly correlated with RVEF and indexed RV mass obtained by ECG-gated CT ($r = -0.71$ and $r = 0.71$, respectively, $p < 0.001$), comparable to invasive hemodynamic measurements (Nagaya et al. 1998). In patients with primary PH referred for lung transplantation, NT-proBNP level at referral was higher in those whose developed RV failure at follow-up (HR = 3.8, 95%CI:2.5–5.8), but this relation lost its significance in multivariate analysis (Dandel et al. 2015) (Table 3).

In the setting of PH secondary to lung parenchymal diseases, NP levels are considerably higher in patients with cor pulmonale than those with isolated COPD (Bando et al. 1999) and have good correlation with hemodynamic findings (Yap et al. 2004) and RV function by echocardiography (RVFAC and RVEF) (Agoston-Coldea et al. 2014). Using TAPSE <16 mm as RV dysfunction criteria, a NT-proBNP cutoff value of 311 pg/ml had 100 % sensitivity (95 % CI: 87.7–100 %) and 84 % specificity (95 % CI: 69.9–93.4 %, AUC = 0.95, $p < 0.0001$). Noteworthy, BMI was significantly higher (31 ± 6 vs. 27 ± 4 kg/m^2, $p = 0.009$) and eGFR significantly lower in the PH group (57 (IQR 23–108) vs. 72 (IQR 69–105) (ml/min^{-1})/1.73 m^{-2}, $p = 0.050$)), two situations having an opposite influence on NT-proBNP level, and this could bias the presented cutoff values (Agoston-Coldea et al. 2014).

In PH, NP levels have great added value, not only for prognostication but also for their capacity to reveal higher RV wall stress as shown consistently by different imaging modalities in multiple situations.

Congenital Heart Diseases

Right heart failure is the final pathway of many congenital heart diseases (CHD) in adulthood caused either by chronic volume or pressure overloads. Consequently, NP have gained a lot of interest in this population. Correlation of NP with imaging in the most commonly encountered congenital heart diseases involving the RV will be reviewed here (Table 4).

Atrial septal defect (ASD) is one of the most frequent CHD potentially leading to RV failure. BNP levels are higher in patients with ASD than in normal controls (42.9 ± 29.4 vs. 8.3 ± 2.6 pg/ml, $p < 0.05$) but remain within normal range (Nagaya et al. 1998; Uz et al. 2011). BNP is lower in patients with ASD than the cutoff for HF diagnostic. This is possibly explained by the younger population in CHD and the chronicity of RV volume overload imposed by the ASD, which could allow for RV adaptation and lessened the expected increase in wall stress. NP levels

Table 3 BNP and NT-proBNP levels, correlations with imaging, and diagnostic accuracies for RV dysfunction and/or dilatation in pulmonary hypertension

First author, year of publication	RV dysfunction definition, nb of patients, mean age, nb of men, mean eGFR, mean BMI	BNP or NT-proBNP median (± SD or IQR) in RV dysfunction group	Controls definition, nb of patients, mean age, nb of men, mean eGFR, mean BMI	BNP or NT-proBNP median (IQR or range) in control group	Correlations with imaging	Cutoff and AUC	Sensitivity and specificity
BNP							
Bando et al. (1999)	28 chronic resp. failure patients: 70.5 years old, 19 men, CKD excluded, no BMI	81.5 ± 13.1 pg/mL	15/28 patients with chronic resp. failure but no cor pulmonale	13.3 ± 2.7 pg/mL	Echo: RVSP: $r = 0.48$, $p = 0.068$	–	–
	13/28 cor pulmonale = RV dilatation or hypertrophy		10 primary lung cancer, 69.2 years old, 7 men	3.5 ± 1.0 pg/mL			
			12 healthy individuals, 29.3 years old, 10 men	7.2 ± 1.0 pg/mL			
Nagaya et al. (1998)	44 patients with RV overload: 18 patients with ASD, 10 with primary PAH and16 with CTEPH, 47 years old, 19 men, no eGFR and BMI Creat >133 µmol/L and significant LV disease excluded	Volume overload patients (ASD): 48 ± 1 pg/mL (mean RVEF 45 %) Pressure overload patients (PAH + CTEPH): 294 ± 72 pg/mL (mean RVEF 31 %)	11 age-matched controls, 50 years old, 5 men	No level given but $p < 0.05$ compared to BNP in patients with volume and pressure overload	ECG-gated CT RVEF: $r = -0.71$, $p < 0.0001$ RVMI: $r = 0.71$, $p < 0.0001$ RVEDVi: $r = 0.12$, p NS RAESVI: $r = 0.33$, p NS	–	–

(*continued*)

Table 3 (continued)

First author, year of publication	RV dysfunction definition, nb of patients, mean age, nb of men, mean eGFR, mean BMI	BNP or NT-proBNP median (± SD or IQR) in RV dysfunction group	Controls definition, nb of patients, mean age, nb of men, mean eGFR, mean BMI	BNP or NT-proBNP median (IQR or range) in control group	Correlations with imaging	Cutoff and AUC	Sensitivity and specificity
NT-proBNP							
Agoston-Coldea et al. (2014)	36 patients with PHT secondary to COPD, (mean RVEF 39.5), 59 years old, men, eGFR 57.1, BMI 31	1322.7 (234–2,567) pg/mL	36 healthy subjects, 59 years old, 18 men, eGFR 71.5, BMI 27	203.5 (69–311) pg/mL		TAPSE <16 mm: 11 pg/ml	Sens. 100 % (95 % CI: 87.7–100 %)
						AUC: 0.945, $p < 0.0001$	Spec. 84.0 % (95 % CI: 69.9–93.4 %)
Dandel et al. (2015)	79 PAH patients ref. for transplant 23/79 will develop RVF: 51 years old, 8 men, creat 1.39 mg/dL, no BMI Mean baseline RVEF 30 % RVF = syst. venous congestion with progressive RV and RA dilation, TR ≥ grade 3 and worsening symptoms	2,690 (645–4,865) pg/ml at baseline	56/79 won't develop RVF: 47 years old, 19 men, creat 1.31 mg/dL, no BMI Mean baseline RVEF 35 %	1,256 (570–2,150) pg/ml at baseline	High NT-proBNP HR 3.8, CI 2.5–5.8, $p < 0.01$ for RVF, but NS in multivariate analysis	–	–

This table summarizes the published studies on the relationship between BNP/NT-proBNP and function and/or size of the RV on imaging in patients with pulmonary hypertension. They are separated between BNP and NT-proBNP for easier comparison

Abbreviation key: *ASD* atrial septal defect, *AUC* area under curve, *BMI* body mass index (kg/m^2), *CKD* chronic kidney disease, *COPD* chronic obstructive pulmonary disease, *creat* creatinine, *CT* computed tomography, *CTEPH* chronic thromboembolic pulmonary hypertension, *echo* echocardiogram, *eGFR* estimated glomerular filtration rate (cc/min/1.73 m^2), *HR* hazard ratio, *IQR* interquartile range, *nb* number, *NS* non significant, *PAH* pulmonary arterial hypertension, *RAESVI* right atrial end-systolic volume indexed (mL/m^2), *ref.* referred, *resp.* respiratory, *RV* right ventricle, *RVEDVI* right ventricle end-diastolic volume indexed (mL/m^2), *RVEF* right ventricle ejection fraction, *RVF* right ventricle failure, *RVMI* right ventricle mass index, *SD* standard deviation, *TAPSE* tricuspid annular plane systolic excursion (mm)

Table 4 BNP and NT-proBNP levels, correlations with imaging, and diagnostic accuracies for RV dysfunction and/or dilatation in selected congenital heart diseases

First author, year of publication	Nb of patients, mean age, nb men	BNP/ NT-proBNP mean ± SD in patients	Controls definition, nb of controls, mean age, nb men	BNP/NT-proBNP mean ± SD in controls	Mean RVEF ± SD in patients, correlation coefficient	Mean RVSP ± SD in patients, correlation coefficient	Mean Qp/Qs ± SD in subjects; correlation coefficient	Mean RVEDV or RVEDD ± SD in subjects; correlation coefficient	Other correlations with imaging
Atrial septal defect									
BNP									
Uz et al. (2011)	56 patients, 22.9 years old, 45 men BMI 22.2 Abnormal LVEF excluded	42.9 ± 29.4 pg/mL	31 healthy volunteers, 22.7 years old, 23 men, BMI 21.8	8.3 ± 2.6 pg/mL	–	Echo: 37.4 ± 9.8 mmHg; $r = 0.61$, $p < 0.001$	Echo: 2.6 ± 0.9; $r = 0.71$, $p < 0.0001$	Echo: RVEDV 83.2 ± 28.7 mL/m^2; $r = 0.55$, $p < 0.001$	MPI by TDI 0.46 ± 0.06; $r = 0.50$, $p < 0.001$ 24.6 pg/ml has sens. 75 % and spec. 66 % for Qp/Qs >1.5
NT-proBNP									
Schoen et al. (2007)	20 patients, 43 years old, 8 men	240 ± 93 pg/mL	Same patients 12 months after ASD closure (percut)	116 ± 62 pg/mL ($p < 0.01$ vs. pre-closure)	CMR: 37 ± 9 % at baseline; $r = 0.22$ (non sign)	Echo: 33 ± 8 mmHg; $r = 0.75$, $p < 0.01$	9/20 had Qp/Qs ≥ 2 $r = 0.62$, $p < 0.05$	Echo RVEDD: 36 ± 4 mm CMR RVEDV: 127 ± 17 mL/m^2; $r = 0.65$, $p < 0.05$	
Weber et al. 2006	12 patients, 44.4 years old, 6 men Mean creat 77.8 ± 32.7 µmol/L (baseline)	87 (IQR: 65–181) pg/mL	Same patients after ASD closure (percut)	9 ± 13 days post-closure: 315 pg/ml (IQR: 133–384) 138 ± 64 days post-closure: 102 pg/mL (IQR: 82–188)	CMR: Baseline: 47.0 ± 6.1 % Early post-closure: 43.6 ± 6.3 % Late post-closure: 50.8 ± 13.8 %	Catheterism: 16.3 ± 6.3 mmHg	Oximetry: 2.10 ± 0.68	CMR: RVEDD; RVEDV baseline: 5.6 ± 7.0 mm; 211 ± 70 mL Early post-closure: 38.6 ± 4.3 mm;	

(*continued*)

Table 4 (continued)

First author, year of publication	Nb of patients, mean age, nb men	BNP/ NT-proBNP mean ± SD in patients	Controls definition, nb of controls, mean age, nb men	BNP/NT-proBNP mean ± SD in controls	Mean RVEF ± SD in patients, correlation coefficient	Mean RVSP ± SD in patients, correlation coefficient	Mean Qp/Qs ± SD in subjects; correlation coefficient	Mean RVEDV or RVEDD ± SD in subjects; correlation coefficient	Other correlations with imaging
				$p < 0.001$ (pre vs. post)	$P = 0.165$ (pre vs. post)			129 ± 37 mL Late post-closure: 36.2 ± 7.9 mm; 139 ± 42 mL $p = 0.001$; 0.015 (pre vs. post)	
Tetralogy of Fallot (post-repair)									
BNP									
Brili et al. (2005)	25 patients, repaired at mean 18.8 years old, 28.4 years old, sex not given	85.0 ± 87 pg/mL	25 healthy controls	5.36 ± 1.0 pg/mL	–	–	Mod to severe in 22 patients	Echo: RVD/LVD 1.00 ± 0.12 $r = 0.521$ $p < 0.01$	TDI Sa: 8.16 ± 1.15 cm/s TDI Ea: 10.00 ± 2.8 cm/s TDI Aa: 5.64 ± 1.77 cm/s All NS
Trojnarska et al. (2006)	60 adults TOF repaired at mean 7.5 years old, 27.6 years old, 29 men	34.8 ± 27.1 pg/mL	28 healthy controls, 28.7 years old, 13 men	11.5 ± 6.5 pg/mL	–	–	Echo: mod. to severe in 34 patients p NS	–	–
NT-proBNP									
Festa et al. (2007)	70 adults TOF repaired at mean 3 years old, 21 years old, 44 men Renal failure excluded	218 ± 30 ng/L	48 healthy subjects age and sex-matched, 22 years old	39 ± 3 ng/L $p < 0.001$ (vs. patients)	CMR: 53 ± 1 % $r = -0.32$, $p < 0.01$	Echo: 50.2 ± 2.8 mmHg $r = 0.27, p < 0.05$	CMR: PVR fraction 28 ± 2 % r = non sign	CMR: RVEDVi 140 ± 5 ml/m^2 $r = 0.40$, $p < 0.001$	CMR: RVESVi >90 ml/m^2: 200 ng/L; AUC 0.758 (95 % CI: 0.605-0.912); sens. 69 %; spec. 74 %

Norozi et al. (2005)	50 adults TOF repaired at mean 6.6–7.3 years old, 27.8 years old, 24 men	Men: 147 ± 28 pg/ml Women: 180 ± 23 pg/ml	100 healthy subjects age- and sex-matched healthy controls	Men: 32 ± 3 pg/ml Women: 43 ± 4 pg/ml	–	Echo: TVR Vmax Men: 2.8 ± 0.2 m/s Women: 2.7 ± 0.1 m/s $r = 0.42, p < 0.01$	Echo: Mod. to severe in 20 patients	Echo: RVEDD Men: 38 ± 1.6 mm Women: 35 ± 1.3 mm $r = 0.45$, $p < 0.05$	–
Systemic right ventricle									
BNP									
Vogt et al. (2009)	16 patients, 25.6 yearsold, atrial switch in all	63.3 ± 47.5 pg/mL	–	–	–	Echo (TEE): IVA 0.7 ± 0.25; s-Velocity 1.8 ± 0.9; e-Velocity 4.3 ± 2.2; Strain 21.5 ± 4.3 All NS at rest	Grade ≥3 in 5/16 patients $r = 0.55$, $p < 0.03$	–	Increase in IVA with dobutamine (0.7 ± 0.25–1.5 ± 0.9) $r = -0.57, p < 0.02$
NT-proBNP									
Dore et al. (2005)	29 patients, 30.3 years old, 83 % men 21 mustard at mean 2.5 years old and 8 ccTGA Creat >250 mmol/L excluded	257.7 ± 243.4 pg/mL	–	–	Echo: 41.6 ± 9.3 % $r = -0.42$, $p = 0.02$	–	Moderate 20 % of patients (no severe)	Echo: mild RV dilatation in 45 % and mod. to severe in 52 % of patients	–
Kozelj et al. (2008)	19 patients, 35 years old, 10 men All ccTGA,	653 ± 1535.2 ng/L	19 healthy controls, 35.4 years old, 10 men	47.9 ± 34.4 ng/L	CMR or CT: 47.86 ± 15.3 % $r = -0.53$, $p = 0.02$	TDI E_t velocity: 14.0 ± 4.5 $r = -0.64$, $p = 0.003$	Moderate in 31.5 % and severe in 10.5 % (5 with Ebstein's) r = NS	CMR or CT: RVEDV 163.63 (range 115–230) $r = 0.50$, $p = 0.026$	–

(*continued*)

Table 4 (continued)

First author, year of publication	Nb of patients, mean age, nb men	BNP/ NT-proBNP mean ± SD in patients	Controls definition, nb of controls, mean age, nb men	BNP/NT-proBNP mean ± SD in controls	Mean RVEF ± SD in patients, correlation coefficient	Mean RVSP ± SD in patients, correlation coefficient	Mean Qp/Qs ± SD in subjects; correlation coefficient	Mean RVEDV or RVEDD ± SD in subjects; correlation coefficient	Other correlations with imaging
Plymen et al. (2010)	35 patients, 29 years old, all had atrial switch, at mean 14 months yo, 24 men	26 (5–135) pmol/L	–	–	CMR: 51 ± 8 % $r = -0.54$, $p = 0.0007$	–	–	CMR: RVEDV indexed 107 ± 27 ml/m^2 $r = 0.43$, $p = 0.01$	–
Other congenital heart diseases									
BNP									
Perlowski et al. (2007)	54 patients with various CHD, 39 years old, 23 men, 78 % cyanotic CHD, 37 with previous surgical repair	–	–	–	RVEF results not given; Cyanotic CHD (22 patients): $r = -0.189$, $p = 0.33$ Non cyanotic CHD (4 patients): $r = 0.102$, $p = 0.811$	–	Results not given (MPI by PWD) Cyanotic CHD (18 patients): $r = 0.743$, $p < 0.0001$ Non cyanotic CHD (5 patients): $r = 0.907$, $p = 0.034$	–	–

This table summarizes the published studies on the relationship between BNP/NT-proBNP and function and/or size of the RV on imaging in patients with congenital heart diseases. They are separated between congenital heart defects and important variables or confounding factors are adapted for each disease

Abbreviation key: *ASD* atrial septal defect, *ccTGA* congenitally corrected transposition of the great arteries, *CHD* congenital heart disease, *CMR* cardiac magnetic resonance, *creat* creatinine, *CT* computed tomography, *echo* echocardiography, *e-Velocity* peak myocardial velocities during early diastole (cm/s), *IQR* interquartile range, *IVA* myocardial acceleration during isovolumic contraction (m/s^2), *LVEF* left ventricle ejection fraction, *MPI* myocardial performance index, *nb* number, *percut.* percutaneous, *PVR* pulmonary valve regurgitation, *PWD* pulsed wave Doppler, *RV* right ventricle, *RVEDD* right ventricle end-diastolic diameter (mm), *RVEDV* right ventricle end-diastolic volume (mL), *RVEF* right ventricle ejection fraction, *RVD/LVD* ratio of right ventricle and left ventricle diameters, *RVSP* right ventricle systolic pressure (mmHg), *SD* standard deviation, *TDI Aa* tissue Doppler imaging peak tricuspid annular velocity during late diastole (cm/s), *TDI E, velocity* tissue Doppler imaging peak tricuspid early diastolic annular velocity (cm/s), *s-Velocity* peak myocardial velocities during systolic isovolumic contraction (cm/s), *TDI Sa* tissue Doppler imaging peak tricuspid annular velocity during systole (cm/s), *TDI Ea* tissue Doppler imaging peak tricuspid annular velocity during early diastole (cm/s), *TEE* transesophageal echocardiogram, *TOF* tetralogy of Fallot, *TVR* tricuspid valve regurgitation

in patients with ASD are correlated with right-sided volumes and function (RV-MPI) by echocardiogram ($r = 0.55$ for volume (Uz et al. 2011); $r = 0.74$ (Perlowski et al. 2007); and $r = 0.50$ (Uz et al. 2011) for function, all $p < 0.001$) and CMR RV volumes ($r = 0.65$, $p < 0.05$ (Schoen et al. 2007)). NT-proBNP also showed good correlation with invasive quantification of the shunt and right-sided pressures ($r = 0.62$ and 0.70, respectively, $p < 0.05$). However, no significant correlations were found between NT-proBNP and RVEF and ASD size (Schoen et al. 2007). Early after ASD closure, LV volume increases, which could also stimulate NP secretion (Weber et al. 2006). Later after closure RVSP by echocardiogram and RVEDV by CMR have good correlation with NT-proBNP reduction ($r = 0.60$ and 0.63, respectively, $p < 0.001$) (Schoen et al. 2007). NP levels correlate with many imaging markers of RV function and size in patients with ASD, and biventricular hemodynamics likely have an important role in NP level variations.

In both operated tetralogy of Fallot and systemic RV, larger RV volumes and lower RVEF are associated with higher circulating levels of NP even in mildly symptomatic patients (mean BNP 34.8–85 pg/ml and mean NT-proBNP 166–218 pg/ml) (Brili et al. 2005; Norozi et al. 2005; Festa et al. 2007; Eindhoven et al. 2012; Trojnarska et al. 2006; Dore et al. 2005). Only one small study failed to find any correlation between NP and echocardiography-derived RV function parameters including strain in 16 adults with systemic RV late after atrial switch surgery. Only improvement of myocardial acceleration during isovolumetric contraction under dobutamine infusion showed statistical correlation in this study ($r = -0.57$, $p < 0.02$) (Vogt et al. 2009). In addition to volumes and RVEF, the severity of pulmonary valvular regurgitation has shown correlation with NP levels in most studies of adult with repaired TOF but one (Trojnarska et al. 2006). Lastly TR can also contribute significantly to further RV volume overload, whether due to associated defects such as Ebstein's anomaly or to the failing systemic RV itself, and its severity correlates with NP levels ($r = 0.55$, $p < 0.03$) (Vogt et al. 2009; Eindhoven et al. 2012).

In conclusion, NP can be useful adjuncts to clinical and imaging assessments of RV function in patients with ASD and complex CHD, but with levels often lower than they would be expected for patients with HF with LV dysfunction.

Arrhythmogenic Right Ventricular Cardiomyopathy

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is caused by fibro-fatty replacement of cardiomyocytes leading to RV dilation and aneurysms, and it is often complicated by clinical right-sided HF. It classically affects predominantly the RV but the LV can also be involved. As for any pathology with RV involvement, CMR remains the gold standard when feasible. Nevertheless, NP levels may have a role in ARVC, with a significant correlation between NT-proBNP and CMR for RVEF ($r = -0.76$) and indexed RV volumes ($r = 0.49$ and $r = 0.70$ for end-diastolic and end-systolic volumes, respectively, all $p < 0.001$) (Cheng et al. 2015). Further, in this same study, a NT-proBNP cutoff of 458 pmol/L was able to discriminate for the

presence of RV dysfunction (RVEF ≤45 % by CMR) with AUC = 0.91 (95 % CI:0.80–0.97, $p < 0.001$) and excellent sensitivity (91 %) and specificity (89 %). Noteworthy, patients with LVEF <55 %, renal impairment, or PH were excluded and the normal upper limit of their NT-proBNP was 400 pmol/L in this study. Hence, these results may not be widely applicable in patients with biventricular dysfunction or other comorbidities.

In ARVC patients with RV dysfunction (mean RVEF = 29 ± 11 %), similar correlation between RVEF and BNP has also been reported using electron-beam CT ($r = -0.59$, $p = 0.025$) with a mean BNP of 61 ± 60 pg/mL compared to 9 ± 6 pg/mL for healthy controls ($p < 0.0001$) (Matsuo et al. 1998). Interestingly, they found positive BNP immunoreactivity in residual myocytes from RV endomyocardial biopsy specimen of all patients with fibro-fatty replacement but not in those without replacement (Matsuo et al. 1998), suggesting increased wall stress on the residual "normal" cardiomyocytes.

In this section, the relationship between the two most clinically used NP BNP and NT-proBNP with different RV imaging modalities was reviewed. They both consistently showed significant association with systolic RV function and, in many clinical entities, with RV volumes. As mentioned, NP levels cannot discriminate between RV and LV origin in heart conditions affecting both ventricles. Nevertheless, both volume and pressure overload can lead to increased RV wall stress and prompt BNP secretion from the RV, a precious adjunct to the challenging imaging of this ventricle. Noteworthy, the range of "abnormal" values observed varied greatly and no absolute cutoff can actually be recommended. Levels varied according to chronicity, compensatory mechanisms, degree of RV fibrosis, and the presence of concomitant valvulopathy and/or LV dysfunction, which could all contribute to NP level variation.

Neutrophil Gelatinase-Associated Lipocalin

Neutrophil gelatinase-associated lipocalin (NGAL) is a glycoprotein expressed by multiple cells including renal tubular cells, endothelial cells, macrophages in atherosclerotic plaques, and cardiomyocytes in failing myocardium (Yndestad et al. 2009). NGAL is an early marker of kidney injury that appears in both the blood and urine within hours in response to renal tubular damage; hence it has been extensively studied in kidney ischemic and nephrotoxic insults. Growing evidence suggests that NGAL may also be involved in processes such as cell survival, inflammation, and matrix degradation in cardiovascular diseases. In HF, NGAL level seems related to disease severity (NYHA class) and NP levels, with potential prognostic value for both survival and hospitalization (Cruz et al. 2012). The comparison between plasma NGAL levels and echocardiographic parameters in patients with stable and decompensated HF led to puzzling results (Shrestha et al. 2011). No correlation could be found with RV S'TDI, LVEF, or invasive

hemodynamic measurements. Unfortunately no detailed RV evaluation was performed in this study. Robust correlation was shown with renal function indices leading to the conclusion that NGAL levels might be determined more by renal function than myocardial remodeling in HF patients. Interestingly, Pronschinske et al. demonstrated higher serum NGAL values in patients who developed clinical RV failure after LVAD implantation (Pronschinske et al. 2014). Since RV failure plays an important role in the cardiorenal syndrome, it would be counterintuitive to not have any relationship between NGAL and RV function. Further data are needed before making a strong case on the relationship between NGAL and RV evaluation, using more precise imaging modalities, such as CMR or CT, and detailed echocardiographic RV parameters such as strain.

sST2

ST2 is an interleukin-1 receptor family member expressed by cardiomyocytes. Two isoforms have been described: ST2L, a transmembrane receptor for IL-33, and soluble ST2 (sST2), which lacks the transmembrane and intracellular domains. Signaling of ST2L/IL-33 is biomechanically activated by cardiomyocytes and has an important role in paracrine-signaling system leading to hypertrophy and cardiac fibrosis. Liaison of IL-33 to the transmembrane ST2L receptor decreases inflammation and LV remodeling (Sanada et al. 2007), while sST2 has opposite action and blocks the beneficial antihypertrophic effects of IL-33. sST2 is believed to act as a decoy receptor for IL-33, preventing its binding to ST2L and therefore impeding cardioprotective effects of ST2L/IL-33 (Sanada et al. 2007). sST2 is one of the most recent biomarkers in cardiology and has been studied in HF, as well as in many other conditions affecting the RV (Dieplinger and Mueller 2015).

Acute Decompensated Heart Failure

sST2 is a predictor of mortality in patients presenting with acute heart failure (Dieplinger and Mueller 2015), independently of NP levels (Weinberg et al. 2003). As for the NP, sST2 serum level is significantly related to LV volumes and ejection fraction but also to RV function. In patients presenting to emergency department for acute dyspnea enrolled in the PRIDE study (Januzzi et al. 2005), multivariate linear regression identified echocardiography-derived RVSP ($t = 2.29, p = 0.002$) and the presence of jugular venous distension ($t = 2.0$, $p = 0.05$) as both independent predictors of higher level of sST2 (Shah et al. 2009b). RV volumes were not a statistically significant predictor, but the prevalence of RV dysfunction was low with 21 % of their population having RV hypokinesis and 15 % RV dilatation and the mean RVFAC was 40 %.

Pulmonary Hypertension

sST2 level was found to be significantly higher in patients with pulmonary arterial hypertension (PAH) compared to normal control subjects (median = 43 ± 19 vs. 15 ± 2 ng/ml, $p < 0.0001$) (Carlomagno et al. 2013). Correlations of sST2 with CMR were also highly significant in this study for all RV parameters (indexed RVEDV $r = 0.623$; indexed RV end-systolic volume $r = 0.75$; RVEF $r = -0.630$; RV mass indexed $r = 0.433$, all $p < 0.05$). Additionally, more patients in the high sST2 group (>43.3 ng/ml) had a large amount of fibrosis at RV insertion points by CMR. Similar results were found for sST2 and RV size by echocardiogram (RVEDD: $r = 0.321$, $p < 0.05$) (Zheng et al. 2014). This supports the hypothesis of the ability of RV cardiomyocytes to release sST2 in response to wall stress, such as chronically elevated RV afterload; nevertheless, an intrinsic role of sST2 in pulmonary arteries remodeling through modulation of the interleukin and endothelin cannot be excluded as a contributory factor (Wang and Wang 2013) (Table 5).

In the setting of PH secondary to lung parenchymal diseases like COPD, different mediators are likely implicated. In this situation, sST2 levels were significantly higher in COPD-PH patients with normal LVEF hospitalized for HF compared to healthy volunteers (1.26, IQR:0.51–2.99 ng/mL and 0.66, IQR:0.16–1.28 ng/mL, respectively, $p < 0.001$) (Agoston-Coldea et al. 2014). Most of the echocardiographic parameters of RV function and size were correlated to sST2, with the highest coefficient for indexed RV volumes ($r = 0.77$, $p \leq 0.001$). When sST2 was compared to NT-proBNP for RV dysfunction detection (defined as TAPSE <16 mm), NT-proBNP had a better sensitivity (100 %; 95 % CI: 88–100 %) using a cutoff value of 311 pg/ml compared to 71.4 % (95 %IC: 51–87 %) for sST2 with a cutoff value of 0.93 ng/ml, but they exhibited a very good and identical specificity (84 %, 95 %CI:70–93 %). AUC was significant for both biomarkers (0.945 and 0.821, respectively, all $p < 0.0001$).

Finally, in ambulatory patients referred for echocardiogram, sST2 level was significantly higher in patients with increased RA and RV volumes and visual RV dysfunction ($\beta = 0.11$, $p = 0.018$), but no correlation was seen between sST2 and LV size or LVEF (Daniels et al. 2010).

sST2 secretion is prompted in cardiomyocytes by wall stress and its level increases in various conditions affecting the RV. Interestingly, the relationship with RV size and function seems to be independent of LV parameters. Unfortunately, there is actually no well-defined cutoff values for RV volumes or RVEF estimation. Furthermore, elevated sST2 levels have been reported in other clinical non-cardiologic situations and the role of sST2 in RV evaluation must be interpreted with caution.

Troponin and High-Sensitivity Cardiac Troponin

Troponins I, T, and C are proteins that regulate calcium-mediated actin and myosin interaction, essential for cardiac muscle contraction and relaxation. Troponin I is expressed only in the cardiac muscle and is therefore specific for cardiac injury.

Table 5 sST2 levels in different pathologies affecting the RV and correlations with imaging

Pathology	First author, publication year	Nb of patients, mean RV function and size	sST2 in patients, median (IQR) or mean ± SD	Nb of controls and definition	sST2 in controls, median (IQR) or mean ± SD	sST2 correlations with RV imaging
Acute heart failure decompensation	Shah et al. (2009b)[a]	134 patients with acute dyspnea; 66 % with acute heart failure RV hypokinesis present in 27 patients and dilation in 19 RVFAC: 40 (32–47) RVSP: 43 (36–54)	0.53 (0.29–1.38) ng/mL	Patients with dyspnea but no heart failure	0.20 (0.12–0.48) ng/mL	Echo: RVFAC $r = -0.175$, $p = 0.05$ TR severity $r = 0.284$, $p = 0.001$ RV area and volumes: NS RVSP: $T = 2.29$, $p = 0.002$ (multivariate)
Pulmonary arterial hypertension	Carlomagno et al. (2013)[b]	25 patients with PAH mPAP: 47 ± 11	42.82 ± 19.09 ng/mL	10 healthy age- and sex-matched controls	14.84 ± 1.90 ng/mL	CMR: RVEDVi: $r = 0.623$, $p = 0.003$ RVESVi: r = 0.754, p < 0.001 RVEF: $r = -0.630$, $p = 0.001$ RVMi: $r = 0.433$, $p = 0.04$
	Zheng et al. (2014)[d]	40 patients with idiopathic PAH RVEDD: 33.5 ± 6.1 mPAP: 60.9 ± 17.6	28.9 ± 13.9 ng/mL	24 healthy volunteers	20.7 ± 7.5 ng/mL	Echo: RVEDD $r = 0.321$, $p = 0.027$

(*continued*)

Table 5 (continued)

Pathology	First author, publication year	Nb of patients, mean RV function and size	sST2 in patients, median (IQR) or mean ± SD	Nb of controls and definition	sST2 in controls, median (IQR) or mean ± SD	sST2 correlations with RV imaging
Pulmonary hypertension secondary to COPD	Agoston-Coldea et al. (2014)[c]	36 patients with PH secondary to COPD RVSP: 57.1 ± 9.8 RV basal diameter: 47.7 ± 16.9 RVEF: 39.5 ± 8.8 % RVFAC: 32.9 ± 7 RV MPI by PWD : 0.55 ± 0.09 TAPSE: 15.0 ± 6.2 S peak velocity: 9.6 ± 2.4 TDI tricuspid E': 9.1 ± 2.2 TDI tricuspid A': 10.7 ± 2.7 E:E': 5.9 ± 2.8	1.26 (0.51–2.98) ng/mL	36 healthy age- and sex-matched volunteers	0.66 (0.16–1.28) ng/mL	Echo: RV basal diam.: $r = 0.661$, $p < 0.001$ RV medial diam.: $r = 0.532$, $p < 0.001$ RV longitudinal diam.: $r = 0.295$, $p = 0.011$ RVEDVi: r = 0.747, $p < 0.001$ RVESVi: $r = 0.772$, $p < 0.001$ TAPSE: $r = -0.773$, $p < 0.001$ S peak velocity: $r = -0.556$, $p < 0.001$ RV MPI PWD: $r = 0.507$, p NS RVFAC: $r = -0.762$ RVEF: $r = -0.799$ $p = 0.001$ TDI tricuspid E': $r = -0.534$, $p < 0.001$ TDI tricuspid A': r = −0.428, $p < 0.001$

						E:E': $r = 0.437$, $p = 0.001$ (all other RV diastolic parameters NS)
Mixed; outpatients referred for echocardiogram	Daniels et al. (2010)[d]	588 outpatients referred for echo 211 patients with sST2 ≥28.25 ng/mL (optimal cutoff for 1 year mortality prediction)	35.6 (31.2–43.6) ng/mL	377 patients with sST2 <211 ng/mL	19.8 (15.8–23.7) ng/mL	Echo: RV hypokinesis: $\beta = 0.11$, $p = 0.18$ (multivariate) RV enlargement NS in multivariate

This table summarizes the published studies on the relationship between sST2 and function and/or size of the RV on imaging. They are separated between pathologies affecting the RV

Abbreviation key: *CMR* cardiac magnetic resonance, *COPD* chronic obstructive pulmonary disease, *echo* echocardiogram, *mPAP* mean pulmonary arterial pressure (mmHg), *MPI* myocardial performance index, *Nb* number, *NS* non significant, *PAH* pulmonary arterial hypertension, *PH* pulmonary hypertension, *PWD* pulsed wave Doppler, *RV* right ventricle, *RVEDD* right ventricle end-diastolic diameter (mm), *RVEDVi* right ventricle end-diastolic volume indexed (mL/m^2), *RVEF* right ventricle ejection fraction, *RVESVi* right ventricle end-systolic volume indexed (mL/m^2), *RVMi* right ventricle mass indexed (g/m^2), *RVFAC* right ventricular fractional area change (%), *RVSP* right ventricle systolic pressure (mmHg), *TAPSE* tricuspid annular plane systolic excursion (mm), *TDI* tissue Doppler imaging (cm/s), *TR* tricuspid regurgitation

[a]sST2 measured with ELISA (Medical and Biological Laboratories Company, Woburn, Mass)

[b]sST2 measured with ELISA (Quantikine, R&D Systems, Abingdon, UK, and Legend-MAX, BioLegend, San Diego, USA)

[c]sST2 measured with ELISA (Human IL-1 sRII – Quantikine ELISA Kit (R&D Systems GmbH, Germany))

[d]sST2 measured with presage ST2 Assay (monoclonal sandwich ELISA, range of 2–200 ng/mL)

Leakage of cardiac troponins can be caused by cardiomyocytes necrosis such as in myocardial infarction, but apoptosis and reversible cardiac cell injury can also release troponins in peripheral blood. Increased wall stress, neurohormonal activation, oxidative stress, inflammatory cytokines, and altered calcium handling could all potentially lead to cardiac troponin leakage (Kociol et al. 2010).The latest generation of troponin assay, high-sensitivity troponin T or I (hsTnT or hsTnI), allows for much higher analytical sensitivity and can detect troponin at a tenfold lower level than previous assays. Troponin levels have powerful prognostic value, not only in acute coronary syndrome but also in multiple other cardiac conditions. The relationship between serum troponin level and RV function on imaging in adults with different clinical conditions affecting the RV will be reviewed here (Table 6).

Heart Failure

Increase in peripheral blood cardiac troponin is a marker of disease severity in patients with HF and signs a worse prognostic (Latini et al. 2007). In 283 consecutive patients with abnormal BNP ($\geq$20 pg/ml), hsTnT was detectable in 98.6 % ($\geq$0.003 ng/mL) in the absence of acute coronary syndrome or atrial fibrillation. Nearly 60 % of patients had a level within the normal range ($\leq$0.014 ng/ml), with a mean level of 0.023 ± 0.032 ng/ml (Kusumoto et al. 2012). The relationship between hsTnT and echocardiographic parameters was investigated and showed significant correlation with RV-MPI by PWD ($r = 0.443$, $p < 0.0001$), which was better than for the LV parameters (LVEF $r = -0.0369$, LV-MPI by PWD $r = 0.37$, and LVEDD $r = 0.242$; all $p < 0.0001$). RV-MPI by PWD persisted as an independently correlated variable in multivariate analysis including LV echocardiographic parameters, age, and eGFR. These findings were not reproduced in a smaller study of 44 patients hospitalized for acute HF with preserved LVEF, but they did not use a high-sensitive assay (Shah et al. 2009a).

Pulmonary Hypertension

Chronically elevated pressures imposed on RV of patients with PH can eventually lead to myocardial injury and troponin leakage in peripheral blood. Furthermore, RV systolic perfusion pressure decreases in advanced PH and diastolic coronary perfusion pressure can be even more reduced causing RV ischemia. RV capillary rarefaction in patients with PH is also observed, contributing to chronic ischemia with destruction of cardiomyocytes (Ryan and Archer 2014). Cardiac troponins are powerful prognostic biomarkers for RV dysfunction and death (Torbicki et al. 2003). While detectable level of hsTnT is found in more than 90 % of patients with PH, it remains associated with death related to RV dysfunction and correlates significantly with basal RV free wall strain and strain rate by echocardiography, but not with MPI (Filusch et al. 2010). Similar results were found with hsTnI in a larger cohort of 255 PH patients from multiple WHO groups, hsTnI being detectable ($\geq$1.2 pg/mL)

Table 6 Cardiac troponins in different pathologies affecting the RV and correlations with imaging

First author, publication year	Nb of patients, mean age, mean eGFR	RV dysfunction definition and nb of patients with RV dysfunction	Troponin mean ± SD or median (IQR or range) in RV dysfunction group	Troponin mean ± SD or median (IQR or range) in normal RV group	Cutoff, AUC, sensitivity and specificity	Correlations with RV imaging
Heart failure						
Kusumoto et al. (2012)	283 patients, 66.5 years old, eGFR 65.9 43 % ischemic cmp	No dysfunction definition Mean RV MPI PWD: 0.36	hsTnT 0.023 ± 0.032 ng/mL	–	–	RV MPI PWD: $r = 0.443$, $p < 0.0001$
Shah et al. (2009a)	44 patients acute HF with LVEF ≥50 %, 74 years old, eGFR 60	No dysfunction definition Mean RVFAC: 43	TnT > 0.01 ng/mL in 19 patients	–	–	RVFAC: $r = -0.2$, p NS RVSP: $r = -0.02$, p NS
Pulmonary hypertension						
Filusch et al. (2010)	55 patients, 53.6 years old, eGFR <60 excluded	No dysfunction definition Mean echo parameters: RVFAC: 24.7, TAPSE: 6.3, RV MPI PWD: 0.58, RV/LV end-diastolic area ratio, 1.4; RV strain: −14.2, RV strain rate: −0.26	hsTnT detectable (≥2 pg/mL) in 90.9 % of patients hsTnT ≥ 13.4 pg/mL in 27.3 % of patients	–	–	RV systolic strain: $r = 0.95$, $p = 0.0018$ RV strain rate: $r = 0.82$, $p = 0.0021$ MPI by PWD: NS
Velez-Martinez et al. (2013)	255 patients, 56 years old, creat 0.9–1.0 mg/dL	No dysfunction definition RVEF per quartile of hsTnI: 55, 50, 45, 44 % RVEDVi per quartile of hsTnI: 141, 149, 166, 181 ml/m^2	hsTnI 6.9 (2.7–12.6) pg/mL, detectable (≥1.2 pg/mL) in 95 % of patients	–	–	CMR: RVEDVi: $r = 0.35$, $p < 0.001$ RVEF: $r = -0.33$, $p < 0.001$; $B = -0.24$, $p < 0.001$ in multivariate

(*continued*)

Table 6 (continued)

First author, publication year	Nb of patients, mean age, mean eGFR	RV dysfunction definition and nb of patients with RV dysfunction	Troponin mean ± SD or median (IQR or range) in RV dysfunction group	Troponin mean ± SD or median (IQR or range) in normal RV group	Cutoff, AUC, sensitivity and specificity	Correlations with RV imaging
Acute pulmonary embolism						
Giannitsis et al. (2000)	56 patients, 68.7 years old, eGFR not given	Echo RV dysfunction: 43/56 Mean Echo RV diameter: 34 mm	TnT ≥0.1 ng/mL in 18/56 RV dysfunction in 100 % of them RV mean diameter: 36 mm	TnT <0.1 ng/mL in 38/56 RV dysfunction in 56 % of them RV mean diameter: 33 mm		
Henzler et al. (2012)	77 patients, 63 years old, CKD in 4/77	RVD if ≥2 echo criteria: dyskinesia or hypokin of RV free-wall, McConnell sign, TAPSE <15 mm, RV/atrial gradient >30 mmHg, RV diameter >30 mm or RV/LV end-diastolic diameter ratio >1 in apical 4-chambers 22/77 patients	TnI 0.179 ± 0.411 g/L	TnI 0.061 ± 0.176 g/L	TnI 0.07 g/L: AUC 0.70 Sens. 67 % Spec. 72 % PPV 56 % NPV 80 %	All correl. with CT RV/LV axial, 4ch and RV/LV volume = NS
Kline et al. (2008)	152 patients, 52–56 years old, eGRF not given	Echo RV hypokinesia (McConnell sign or qualitative hypokin.): 37/152 patients Echo RV dilatation: 36/152 patients	TnI 0.3 (0.0–1.1) ng/mL TnI 0.0 (0.0–0.3) ng/mL	TnI 0.0 (0–0) ng/mL	TnI 0.1 ng/mL: AUC 0.71 Sens. 68 % Spec. 73 % LR+ 2.5	–

Laiho et al. (2012)	63 patients, 55 years old, eGFR not given	CTPA RV/LV diameter >1 and/or interventricular septum deviation 37/63 patients	TnT >0.03 ug/L in 9/37 patients with RVD	TnT >0.03 ug/L in 2/26 patients with no RVD	–	–
Ozsu et al. (2010)	108 patients, 70 years old, eGFR not given	CTPA RV/LV dimension ≥1.1 48/108 patients Echo RV end-diastolic diameter >30 mm 44/108 patients Echo RV dysfunction 48/108 patients	TnT 0.01 (0.01–0.33) ng/mL	TnT 0.01 (0.01–0.24) ng/mL	–	–
Vuilleumier et al. (2008)	50 patients, 74 years old, median creat 87 umol/L	RV/LV diameter ratio >1.0 on CTPA 23/50 patients	TnI 0.032 (0–0.223) ng/mL	TnI 0 (0–0) ng/mL	TnI 0.09 ng/mL AUC: 0.742 Sens.: 43.5 % Spec.: 85.2 % PPV: 71 % NPV: 64 %	RV/LV diameter: $r = 0.25$, p NS

This table summarizes the published studies on the relationship between troponins and function and/or size of the RV on imaging. They are separated between pathologies affecting the RV

Abbreviation key: *AUC* area under curve, *CKD* chronic kidney disease, *cmp* cardiomyopathy, *CMR* cardiac magnetic resonance, *creat* creatinine, *CTPA* computed tomography pulmonary angiography, *echo* echocardiogram, *eGFR* estimated glomerular filtration rate (cc/min/1.73 m^2), *hs* high sensitivity, *IQR* interquartile range, *LR+* positive likelihood ratio, *LV* left ventricle, *MPI* myocardial performance index, *nb* number, *NPV* negative predictive value, *NS* non significant, *PPV* positive predictive value, *PWD* pulsed wave Doppler, *RV* right ventricle, *RVD* right ventricle dysfunction, *RVEF* right ventricle ejection fraction, *RVEDD* right ventricle end-diastolic diameter (mm), *RVEDVi* right ventricle end-diastolic volume indexed (mL/m^2), *RVFAC* right ventricular fractional area change (%), *RVSP* right ventricle systolic pressure (mmHg), *sens.* sensitivity, *SD* standard deviation, *spec.* specificity, *TAPSE* tricuspid annular plane systolic excursion (mm), *TnI* troponin I, *TnT* troponin T, *4ch* 4-chambers view

in 95 % and mortality significantly associated with increasing level (median 6.9 pg/mL, IQR:2.7–14.1 pg/mL) (Velez-Martinez et al. 2013). Higher quartiles of hsTnI were significantly associated with RVEDV and RVEF by CMR ($r = 0.35$ and -0.33, respectively, $p < 0.001$), but not with LVEF, LV volume, and pulmonary capillary wedge pressure. Multivariable linear regression model including age, creatinine, sex, and hemodynamic parameters revealed a significant inverse correlation between hsTnI and RVEF.

Acute Pulmonary Embolism

Cardiac troponins have been studied extensively in the context of acute pulmonary embolism (PE) and are clearly associated with mortality. In a cohort of patients with acute PE including those in shock, all patients with elevated troponin T level ($\geq$0.1 ng/mL) had RV dysfunction by echocardiography (Giannitsis et al. 2000). However, more than half of those with lower level also exhibited RV dysfunction. A higher prevalence of RV dysfunction in patients with elevated troponins T and I (near 50 % of patients with troponin T > 0.1 ng/mL and troponin I > 1.5 ng/mL) was also seen (Konstantinides et al. 2002), but conflicting results have been published, some authors failing to find any correlation (Vuilleumier et al. 2008; Ozsu et al. 2010; Laiho et al. 2012), while others described a moderate diagnostic accuracy for RV dysfunction on echocardiogram (troponin I thresholds of 0.09 and 0.1 ng/mL; AUC = 0.71) (Kline et al. 2008; Henzler et al. 2012). Noteworthy, the majority of these studies did not use a high-sensitivity troponin assay available clinically nowadays.

Potential Applications to Prognosis, Other Diseases, or Conditions

RV evaluation can be quite challenging with difficult echocardiographic windows and contraindications to CMR. Some biomarkers have demonstrated good correlation with RV imaging but there is still a lot more to explore:

- In patients with heart failure, could NP serial measurements correlate with progressive RV deterioration independent of LV function? In that case, could the predictive value of NP be explained at least partially by progressive RV failure?
- Galectin-3 and sST2 are involved in cardiac fibrosis signalling pathway. Could they correlate with RV dilatation and dysfunction progression in diseases with significant RV remodeling such as ARVC and systemic RV?
- Cystatin C and NGAL are markers of kidney function. Since RV function plays a critical role in cardiorenal syndrome, could these biomarkers relate to RV function independently of the LV function?

- Imaging of the RV is improving and few studies compared biomarkers with more recent tools such as three-dimensional echocardiography, strain, and strain rate. The relationship between biomarkers and RV function could be better defined by using these more precise instruments in future studies.
- The use of imaging in combination with multiple biomarkers targeting different RV hemodynamic and remodeling process could be a more powerful tool to evaluate RV size and function. Some literature is already available but often does not include newer imaging modality and biomarkers.

Summary Points

- The role of biomarkers in RV evaluation should be seen as a complement more than a substitute to imaging modalities for most conditions affecting the right heart.
- There is no widely established or accepted cutoff for any biomarker to identify or quantify RV dysfunction or dilatation on imaging.
- Cystatin C is a promising marker of RV dysfunction and remodeling in patients with pulmonary arterial hypertension. It might also correlate with RV function in patients with systolic heart failure.
- Galectin-3 and NGAL have been compared to RV imaging in only very few studies with borderline or negative results.
- BNP and NT-proBNP show significant correlation with RV imaging in acute pulmonary embolism and pulmonary hypertension from multiple etiologies. They have a meaningful potential to identify RV dysfunction in addition of being powerful prognostic biomarkers in these situations.
- BNP and NT-proBNP correlate significantly with RV dysfunction and dilatation in patients with various congenital heart diseases affecting the right heart. Caution should be applied when interpreting NP results in this population since lower levels might be expected than those usually used for heart failure diagnosis and prognosis.
- sST2 correlates with RV size and function in pulmonary hypertension. It may also relate in some extent to RV function in decompensated heart failure, but this needs confirmation in further studies.
- Cardiac troponins are associated with RV dysfunction on imaging in patients with pulmonary hypertension. However, in the context of acute pulmonary embolism, relation between troponins and RV on imaging is mitigated, even if they are both associated with increased mortality.

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Part VI

Resources

Recommended Resources on Biomarkers in Cardiovascular Disease

52

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Contents

Abstract

Cardiovascular disease biomarkers are substances that indicate the presence of cardiovascular disease. They may be released from the heart or result from a specific response to the cardiovascular disease.

Genetic, epigenetic, proteomic, glycomic, and imaging biomarkers can be used for the diagnosis, prognosis, and epidemiology of cardiovascular disease. Ideally, such biomarkers can be assayed in biofluids like blood, which may be collected relatively easily.

Conventional risk scores based on the presence of major cardiovascular risk factors (e.g., age, gender, cigarette smoking history, and hypertension) are widely

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4_52

available. These traditional algorithms have been updated and enhanced by the inclusion of cardiovascular disease biomarkers. The use of biomarkers facilitates the management of cardiovascular disease.

Several potentially relevant novel cardiovascular biomarkers have been discovered through omic technologies such as genomics, and proteomics. It is difficult even for experienced scientists and clinicians to remain up-to-date with the rapid pace of the developments in this field. For those new to the field, it is difficult to know which of the myriad of available sources are reliable. To assist our colleagues, we have therefore produced tables containing reliable, up-to-date resources in this chapter. The experts who assisted with the compilation of these tables of resources are acknowledged below.

Abstract

Biomarkers are of significant value in modern cardiology.

Keywords

Biomarkers • Cardiovascular disease • Evidence • Resources • Books • Journals • Regulatory bodies • Professional societies

Key Points

- Biomarkers are of significant value in modern cardiology.
- Biomarkers are used in screening for cardiovascular disease.
- Biomarkers are used in risk stratification, diagnosis, prognostication, directing initial therapy, monitoring response to treatment, and guiding the choice of further treatments.
- This chapter lists the most up-to-date resources on the regulatory bodies, journals, books, professional bodies, and websites that are relevant to an evidence-based approach to the use of biomarkers of cardiovascular disease.

Introduction

Biomarkers were defined as "characteristics that are objectively measured and evaluated as indicators of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention or other health care intervention" by a consensus panel at the beginning of this millennium (Atkinson et al. 2001). Biomarkers have significant scientific and clinical value in cardiovascular diseases which are common causes of morbidity and mortality. For example, the World Health Organization has indicated that on an annual basis there are 17.5 million deaths from cardiovascular disease. This contributes to one third of all global deaths.

A cardiovascular disease biomarker is a substance that indicates the presence of cardiovascular disease. It could be released from the heart or result from a specific

response to the cardiovascular disease. Genetic, epigenetic, proteomic, glycomic, and imaging biomarkers can be used for the diagnosis, prognosis, and epidemiology of cardiovascular disease. Ideally, such biomarkers can be assayed in noninvasively collected biofluids like blood or serum (Atkinson et al. 2001).

Biomarkers can be used to predict the risk of cardiac disease, diagnose cardiovascular disease after an event, suggest the likely outcome (prognosis) in the absence of treatment, and predict the likely response to treatment. Thus there are four main uses for biomarkers in cardiovascular disease:

1. Risk stratification
2. Diagnosis
3. Prognostication
4. Monitoring response to treatment

Conventional risk scores based on the presence of major cardiovascular risk factors such as age, gender, cigarette smoking history, and hypertension are available. While challenges exist in translating research on novel biomarkers into clinical practice, several biomarkers are currently used routinely by cardiologists (doctors who specialize in the management of patients with cardiovascular disease). For example, these traditional algorithms have been updated and enhanced by the inclusion of physiological biomarkers (e.g., serum lipid and glucose) and biomarkers of cardiac damage (e.g., troponin I and troponin T) and hormonal biomarkers (e.g., brain natriuretic peptide) that are associated with cardiovascular disease. These biomarkers increase the accuracy of risk stratification and facilitate clinical decision making in the management of cardiovascular disease.

Several potentially relevant cardiovascular biomarkers have been discovered through omic technologies such as genomics and proteomics. The use of emerging high-throughput technologies to integrate biomarkers into clinical practice will allow "personalization" of disease management in the future.

It is now difficult even for experienced scientists and clinicians to remain up-to-date. For those new to the field, it is difficult to know which of the myriad of available sources are reliable. To assist colleagues who are interested in understanding more about biomarkers of cardiovascular disease, we have therefore produced tables containing reliable, up-to-date resources in this chapter. The experts who assisted with the compilation of these tables of resources are acknowledged below.

Examples of the definitions, measurement, and applications of biomarkers can be found in this book and also via the recommended resources in the tables below.

Tables 1–**7** list the most up-to-date information on the regulatory bodies (Table 1), professional bodies (Table 2), journals on cardiovascular disease (Table 3), journals on biomarkers (Table 4), books on biomarkers (Table 5), books on cardiovascular disease (Table 6), Emerging techniques and platforms (Table 7), and websites (Table 8) that are relevant to an evidence-based use of biomarkers in cardiovascular disease.

Table 1 Regulatory bodies and organizations. This table lists the regulatory bodies and organizations involved with various aspects of biomarkers

Biomarkers Consortium	www.biomarkersconsortium.org
Biomarker, Imaging, and Quality of Life Studies Funding Program, National Cancer Institute, USA.	www.cancer.gov/aboutnci/organization/ccct/funding/BIQSFP
Biomarker Qualification Program, US Food and Drug Administration	www.fda.gov/Drugs/DevelopmentApprovalProcess/DrugDevelopmentToolsQualificationProgram/ucm284076.htm
Centers for Disease Control and Prevention (CDC)	www.cdc.gov/globalhealth/countries/egypt
Egyptian Society of Cardiology	www.cardioegypt.com
European Medicines Agency	www.ema.europa.eu/ema/index.jsp?curl=pages/special_topics/general/general_content_000349.jsp
International Federation of Clinical Chemistry and laboratory Medicine (IFCC)	www.ifcc.org
Medicines and Healthcare products Regulatory Agency (MHRA)	www.mhra.gov.uk
National Heart Institute, Egypt	www.nhi-egypt.webs.com/nhi.htm
National Institutes of Health	www.nlm.nih.gov/medlineplus/ency/anatomyvideos/000023.htm

Table 2 Professional societies. This table lists the professional societies involved with biomarkers and/or cardiovascular disease

American College of Cardiology	www.acc.org
American Heart Association	www.heart.org
Canadian Cardiovascular Society	www.ccs.ca
European Society of Cardiology	www.escardio.org
Engineering in Medicine and Biology Society	www.embs.org
Italian Federation of Cardiology	www.federcardio.it
Nitric Oxide Society	www.nitricoxidesociety.org

Table 3 Journals publishing on cardiovascular disease. This table lists the top 25 journals publishing original research and review articles related to cardiovascular disease. The list was generated from SCOPUS (www.scopus.com) using general descriptors. The journals are listed in descending order of the total number of articles published in the past 5 years. Of course, different indexing terms or different databases will produce different lists, so this is a general guide only. For example, journals associated with biomarker discovery will produce a different list (see Table 4)

International Journal of Cardiology
Plos One
Journal of the American College of Cardiology
Circulation
Annals of Thoracic Surgery
American Journal of Cardiology
Journal of Thoracic and Cardiovascular Surgery
Stroke
European Heart Journal
Catheterization and Cardiovascular Interventions
BMJ Case Reports
Heart
Heart Rhythm
Hypertension
Circulation Journal
Arteriosclerosis Thrombosis and Vascular Biology
European Journal of Cardio Thoracic Surgery
Atherosclerosis
Circulation Research
New England Journal of Medicine
Resuscitation
American Journal of Physiology Heart and Circulatory Physiology
Interactive Cardiovascular and Thoracic Surgery
Europace
American Heart Journal

Table 4 Journals publishing on cardiovascular disease and biomarkers. This table lists the top 25 journals publishing original research and review articles related to cardiovascular disease and biomarkers. The list was generated from SCOPUS (www.scopus.com) using general descriptors. The journals are listed in descending order of the total number of articles published in the past 5 years. Of course, different indexing terms or different databases will produce different lists, so this is a general guide only

Plos One
International Journal of Cardiology
Atherosclerosis
Journal of the American College of Cardiology
American Journal of Cardiology
Arteriosclerosis, Thrombosis and Vascular Biology
Circulation
Stroke
European Heart Journal
Heart
Hypertension
Circulation Journal
Cardiovascular Diabetology
Circulation Research
European Journal of Heart Failure
Clinica Chimica Acta
American Heart Journal
Journal of Thoracic and Cardiovascular Surgery
Clinical Chemistry
Nephrology Dialysis Transplantation
Journal of Cardiology
Journal of Hypertension
Journal of Clinical Endocrinology and Metabolism
Clinical Biochemistry
Nutrition Metabolism and Cardiovascular Diseases

Table 5 Relevant books on biomarkers. This table lists books on biomarkers

Aptamers in Bioanalysis. Mascini M. Wiley-Interscience, 2009, USA.
Handbook of Biomarkers. Kewal KJ. Lippincott, 2010, USA.
Biomarker Guide. Peters KE, Walters CC, Moldowan JM. Cambridge University Press, 2010, USA.
Aptamer Handbook: Functional Oligonucleotides and Their Applications. Klussmann S (editor). Wiley-VCH, 2006, Weinheim.

Table 6 Relevant books on cardiovascular disease. This table lists books on cardiovascular disease

Braunwald's Heart Disease: A Textbook of Cardiovascular Medicine, 10th Edition. Mann DL, Zipes PD, Libby P, Bonow RO (editors). Elsevier, 2014, USA
Cardiac remodelling. Mechanisms and Treatment. Greenberg B. Taylor & Francis Group, 2006, USA.
Cardiovascular Genetics and Genomics. Roden D. Wiley-Blackwell, American Heart Association, 2009, USA.
ECG Diagnosis in Clinical Practice. Vecht R, Gatzoulis MA, Peters N. Springer-Verlag, 2009, UK.
Electrical Diseases of the Heart. Genetics, Mechanisms, Treatment, Prevention. Gussak I, Antzelevitch C, Wilde AAM, Friedman PA, Ackerman MJ, Shen W. Springer-Verlag, 2008, UK.
Handbook of Emergency Cardiovascular Care for Healthcare Providers (American HA Handbook of Emergency Cardiovascular Care). Hazinski MF, Samson R, Schexnayder S (Editors). AHA, 2010, USA.
Heart Failure: A Companion to Braunwald's Heart Disease, 3rd Edition. Mann DL, Felker MG (editor) Elsevier, 2016, USA.
Manual of Research Techniques in Cardiovascular Medicine. Ardehali H, Bolli R, Losordo DW. Wiley Blackwell, 2014, UK
Pathophysiology of Heart Disease. Lilly LS. Wolters Kluwer, 2014, USA.
Physiology of the heart. Katz AM. Wolters Kluwer, 2011, USA

Table 7 Sources and resources for emerging techniques and platforms. This table lists some emerging sources, resources platforms in biomarker discovery and application

Biobanking and Biomolecular Resources Research Infrastructure	www.bbmri.eu
University of Zurich Progenetix database	www.progenetix.org/cgi-bin/pgHome.cgi

Table 8 Relevant Internet resources. This table lists some Internet resources on biomarkers and cardiovascular disease

Biomarkers Test (BMT)	www.biomarkers.it
Biomed Central (BMC) Biomarkers	www.biomarkerres.org
CardioAlex 2013	www.boehringer-ingelheim.com/mena/media/press_releases/cardio_alex_2013.html
Medscape	www.medscape.com
News medical	www.news-medical.net/health/What-is-a-Biomarker.aspx
Pro-CNIC Foundation (Fundación ProCNIC)	www.fundacionprocnic.es
Spanish Cardiovascular Research Network (Red de Investigación Cardiovascular: RIC)	www.redcardiovascular.com
Spanish National Cardiovascular Research Center (Centro Nacional de Investigación Cardiovascular: CNIC)	http://www.cnic.es
Texas Heart Institute	www.texasheart.org/HIC/Anatomy/index.cfm
Theheart.org	www.theheart.org
World Health Organization	www.who.int/mediacentre/factsheets/fs297/en/index.html

Acknowledgments We would like to thank the following authors for contributing to the development of this resource:

Anderson K, Bashford G, Carnero A, Clarke S, De Rossi A, Diakos C, Gad M, Nistal JF, Noh D-Y, Schraml PH, Staikou C, Takenaka S, Tomai F, Wonshik H, and Yi L.

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V.B. Patel, V.R. Preedy (eds.), *Biomarkers in Cardiovascular Disease*, Biomarkers in Disease: Methods, Discoveries and Applications, DOI 10.1007/978-94-007-7678-4