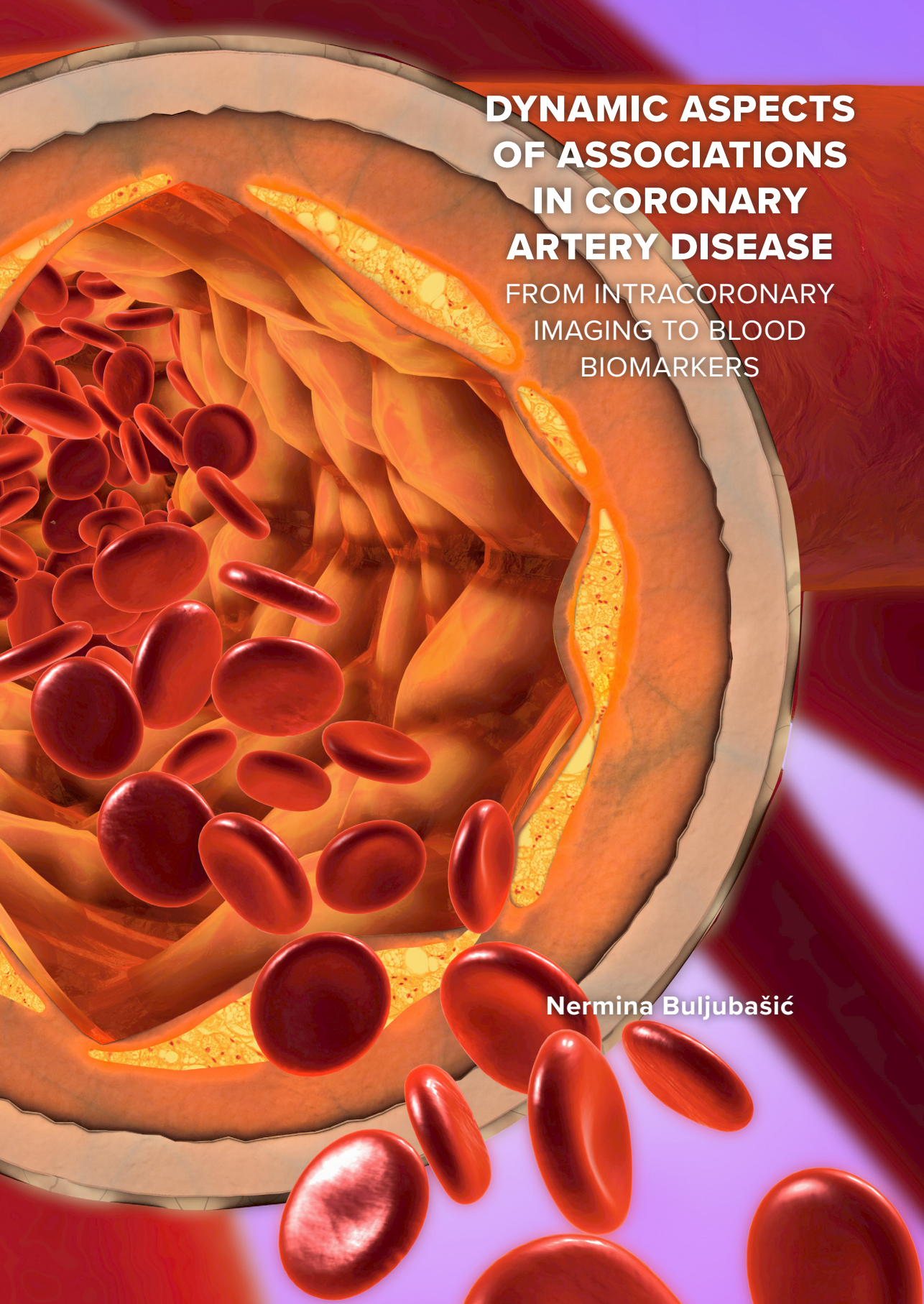


DYNAMIC ASPECTS OF ASSOCIATIONS IN CORONARY ARTERY DISEASE

FROM INTRACORONARY IMAGING TO BLOOD BIOMARKERS

Nermina Buljubašić

Dynamic Aspects of Associations in Coronary Artery Disease: From Intracoronary Imaging to Blood Biomarkers

Nermina Buljubašić

Financial support for the publication of this thesis is gratefully acknowledged and was generously provided by: ABN Amro Bank, Boehringer Ingelheim, Cardialysis, ChipSoft, Dutch Heart Foundation (Nederlandse Hartstichting), Erasmus Medisch Centrum (Thoraxcentrum), Erasmus Universiteit Rotterdam and Servier Nederland Farma B.V.

Cover design: Ilse Modder - graphic designer, Noordwijkerhout, the Netherlands
Lay-out design: Ilse Modder - graphic designer, Noordwijkerhout, the Netherlands
Printing: Gildeprint B.V. - Enschede, the Netherlands

ISBN: 978-94-6402-038-0

Dynamic Aspects of Associations in Coronary Artery Disease: From Intracoronary Imaging to Blood Biomarkers

Dynamische aspecten van associaties in coronair vaatlijden:
van intracoronaire beeldvorming tot bloedbiomarkers

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus
Prof. dr. R.C.M.E. Engels
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 29 januari 2020 om 11:30 uur.

Door

Nermina Buljubašić
geboren te Loznica

Erasmus University Rotterdam

Erasmus

Promotiecommissie

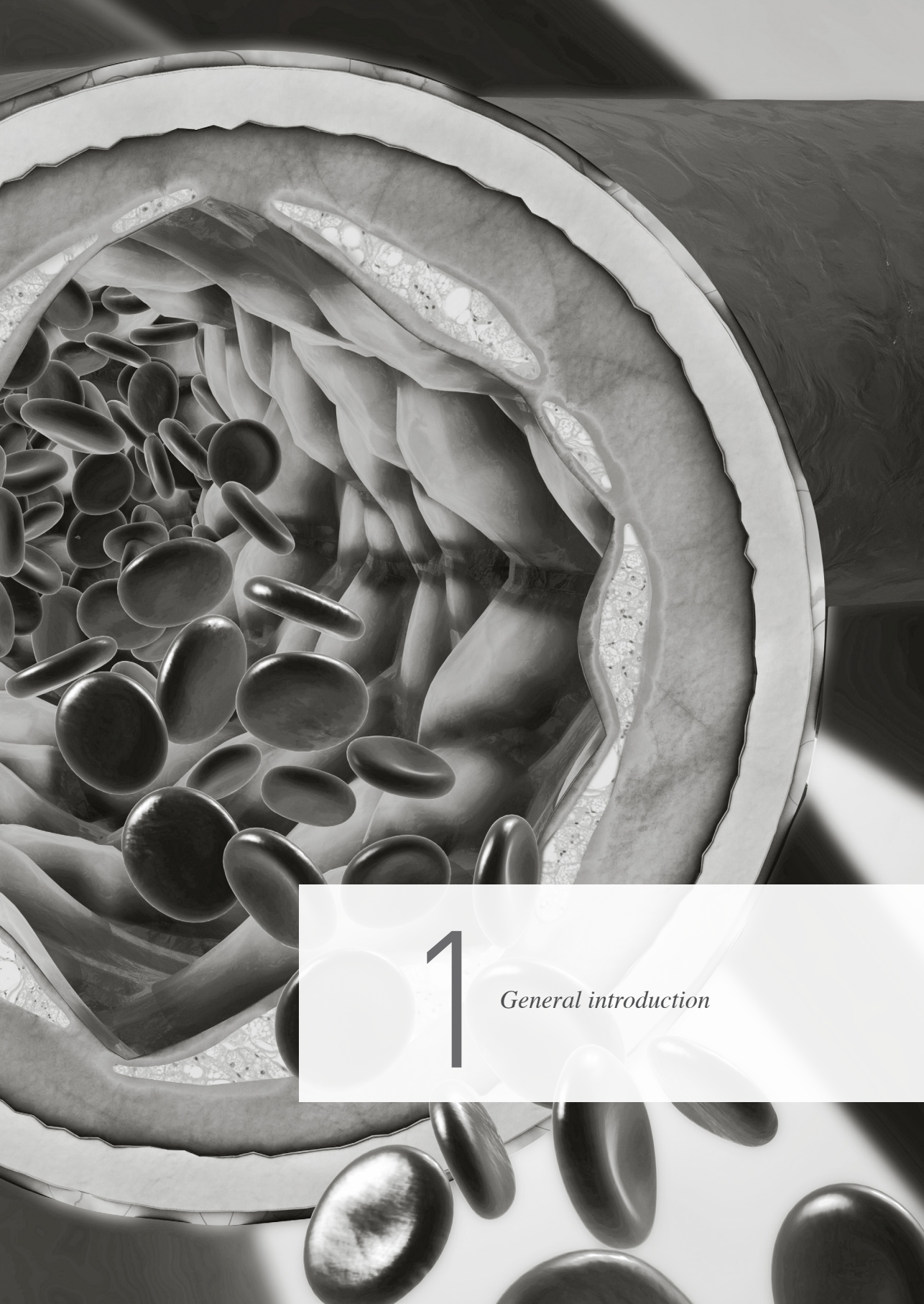
Promotor:	Prof. dr. ir. H. Boersma
Commissieleden:	Prof. dr. R.H.N. van Schaik, Erasmus Medisch Centrum
	Prof. dr. F.W. Asselbergs, Utrecht Medisch Centrum
	Prof. dr. R.J. de Winter, Amsterdam Medisch Centrum
	Dr. J.E. Roeters van Lennep, Erasmus Medisch Centrum
	Prof. dr. F. Zijlstra, Erasmus Medisch Centrum
Copromotoren:	Dr. I. Kardys
	Dr. K.M. Akkerhuis

Financial support by the Dutch Heart Foundation for the publication of this thesis is gratefully acknowledged.

Funded by Hartstichting

Table of contents

Voor mijn ouders
Mojim roditeljima



1 *General introduction*

1 General introduction

Worldwide impact of coronary artery disease: how serious is it?

For decades coronary artery disease (CAD) has been the most common manifestation of cardiovascular disease and leading cause of death worldwide [1, 2]. According to the 2017 Global Burden of Disease Study, CAD affected 126.5 million people [1] in that year and resulted in 8.9 million deaths [2], which is 15.9% of all deaths. Although temporal analyses from population-based epidemiological data have shown favorable trends with declining CAD mortality rates in economically developed (Western) areas of Europe [3] and the United States [4] over the past decennia, developing (non-Western) regions nowadays encounter a substantial rise in CAD burden [5, 6]. All in all, CAD has remained and continues to be an enormous disease burden worldwide.

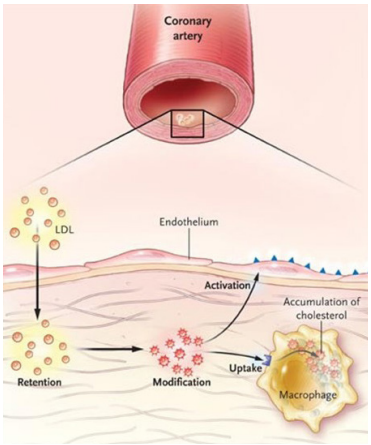
Coronary artery disease: a manifestation of atherosclerosis

Coronary atherosclerosis is the underlying multifactorial pathophysiological process that eventually leads to CAD. The pathogenesis of atherosclerosis involves multiple molecular mediators, influenced by both environmental (risk) factors and genetic predisposition. These determinants subsequently promote a sequence of events leading to atherosclerotic plaque progression, ultimately resulting in a clinical event.

Determinants of atherosclerosis

In a healthy coronary artery, the lumen along the inner side of a vessel wall is covered with a monolayer of endothelial cells. The endothelium possesses several functions and is involved in many biological processes regulating vascular homeostasis, including blood coagulation, vessel tone regulation and controlling the passage of components out of the bloodstream [7]. Endothelial dysfunction occurs at prone areas where the endothelial cell layer is injured by (external) stimuli, such as for example toxic substances in cigarette smoke, endotoxins or blood flow disturbances. A leaky, activated and dysfunctional endothelium leads to a series of lipid-driven immunoinflammatory and fibroproliferative responses and is an important initial step in the manifestation of atherosclerosis [7]. By expressing adhesion molecules

on the surfaces of injured endothelial cells, leukocytes (most importantly monocytes and T-lymphocytes) are recruited and captured [7, 8]. Also, changes in endothelial permeability promote plasma molecules and lipoprotein particles to pass from the lumen through a defective endothelium into the subendothelial space. Specifically, low-density lipoprotein (LDL) particles get entrapped and modified (i.e. oxidized) [9]. Oxidized LDL is one of the most important atherogenic chemoattractants, meaning that chemokines are attracted to stimulate transendothelial migration of the attached leucocytes on the endothelium. Once differentiated into macrophages, monocytes produce pro-inflammatory cytokines (e.g. TNF-α) [10] and facilitate phagocytosis of oxidized LDL, leading to the development of lipid-laden macrophages becoming 'foam cells' [8]. Consequently, these foam cells undergo apoptosis and necrosis with a release of even more lipids, cytokines and prothrombotic molecules that locally accumulate, leading to the formation of a necrotic lipid core within the intima [11].



Formation of 'foam cells'.
Image adapted from Hansson et al. N Engl J Med 2005; 352: 1685-1695.

In response to this biochemical outburst reaction, smooth muscle cells from the vascular wall are activated as well to proliferate and synthesize extracellular matrix proteins [11]. In conclusion, endothelial dysfunction initiates a series of biochemical reactions, creating a local pro-atherogenic environment.

Risk factors act as irritative stimuli at several points in this pathogenic pathway, aggravating the underlying processes. Cigarette smoking impacts all phases of atherosclerosis and is one the most important risk factors for CAD worldwide [12]. For example, the toxic components of cigarette smoke contribute to atherosclerosis by mediating endothelial dysfunction, enhancing oxidative modification of LDL, stimulating pro-inflammatory cytokines leading to increased leukocyte recruitment and inducing a prothrombotic state in the coronary arteries [13]. Lipid abnormalities (dyslipidemia), in particular elevated levels of LDL, form another important CAD risk factor [12]. A direct excessive supply of circulating amounts of lipoproteins, that are retained, accumulated and subsequently modified in the atherosclerotic plaque, mainly triggers a local inflammatory response [14]. A state of chronic hyperglycemia in diabetes mellitus (type 2) is related to dyslipidemia, partly explaining the strong association between diabetes and CAD [12]. Besides the impact of hyperglycemia on

dyslipidemia, it causes oxidative stress and thereby plenty of other pro-atherogenic responses including enhancement of endothelium dysfunction, inflammation and thrombogenicity [15]. Lastly, hypertension might also lead to atherosclerosis and is considered to be a major risk factor as well for the risk of CAD [12]. Since hypertension causes increased arterial wall thickness, it is hypothesized that a larger diffusion distance from the lumen for oxygen is created, probably leading to increased concentrations of free radicals (oxidative stress) [16]. Consequently, endothelial injury is induced that promotes leukocyte recruitment and smooth muscle cell proliferation. Against this background of external influences on the initiation and development of atherosclerosis, underlying synergistic interactions with genetic determinants steer all processes into a certain direction. In all biological pathways of atherogenesis, many regulatory genes are identified to be involved and thus largely determine an individual's susceptibility to CAD [17].

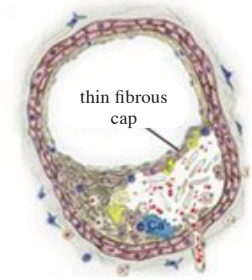
Altogether it can be concluded that the underlying pathophysiological processes, its interplay with environmental risk factors and involvement of many genes, make atherosclerosis a very complex disease.

Coronary atherosclerotic plaque progression: from initiation to disease

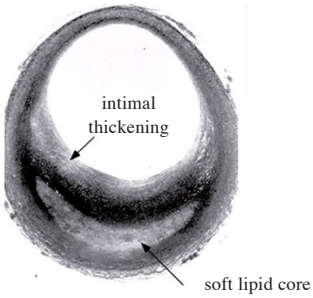
The previously mentioned cell types contribute to coronary atherosclerotic plaque progression. Under the driving influence of risk factors, an atherosclerotic plaque progresses through multiple stages: from early 'fatty streaks' to 'thin-cap fibroatheromas', giving rise to various clinical expressions of CAD (Figure 1).

The earliest coronary lesion histologically is the 'fatty streak', which starts as focal thickening of the vascular wall intima layer with accumulation of primarily macrophage foam cells, along with smooth muscle cells [18]. As these lesions expand ('pathological intimal thickening'), extracellular lipid pools are formed underneath layers of smooth muscle cells in a proteoglycan-rich matrix with affinity for plasma lipoprotein particles [18, 19]. Typically, these lesions contain a deeply located soft lipid core and an absent necrotic core. As this stage further progresses, 'fibrous cap atheromas' are formed, which are all identified by fibrous cap development [20]. A fibrous cap is a distinct layer of connective tissue covering a necrotic, fatty mass and is usually crucial for maintaining plaque integrity and stability. Depending in which direction the plaque has progressed and what the prevailing compound of the plaque is, distinct types of fibrous cap atheromas can be discerned. The 'classic' fibroatheroma plaque is defined as a lesion with a necrotic, fatty core and a thick cellular

fibrous cap. When the lesion contains a remarkable amount of collagen-rich fibrous tissue and little lipid, the lesion is classified as a 'fibrotic' lesion. If the fibrous cap encapsulates mainly calcified tissue, the lesion is referred to as a 'fibrocalcific' lesion. The final and most critical stage of atherosclerotic plaque progression is the 'thin-cap fibroatheroma' (TCFA), which is characterized by a relatively large eccentric necrotic core and an overlying thin fibrous cap (< 65μm), infiltrated by macrophages [20]. This specific lesion type is vulnerable for disruption of the fibrous cap. Once the fibrous cap ruptures, the thrombogenic material is exposed to the circulating blood and a coagulation cascade is activated, leading to formation of luminal thrombosis.



Thin-cap fibroatheroma.
Image adapted from Bentzon et al.
Circ Res 2014; 114:1852-1866.



Pathological intimal thickening.
Image adapted from
© 2019 UpToDate, Inc.

A couple of mechanisms are believed to be responsible for the dynamic progression of atherosclerotic plaque growth and destabilization. First, human autopsy studies have identified intraplaque hemorrhage as a common repetitive feature in advanced coronary atherosclerotic lesions [21]. This treat results from neovascularization, leading to fragile, disrupted, leaky microvessels with extravasation from red blood cells within the atherosclerotic plaque. Another common source of intraplaque hemorrhage is direct entrance of blood into the plaque from the lumen through a plaque fissure. Not only it causes episodic plaque growth, but also triggers a cellular response involving plaque infiltration by cholesterol and inflammatory cells, which in turn leads to plaque instability [21, 22]. A major protective mechanism against direct toxic effects of haemoglobin, released from the red blood cells within the atherosclerotic plaque, is the presence of circulating haptoglobin, whose major function is to bind excess haemoglobin [23]. A second mechanism through which atherosclerotic plaques progress is

through subclinical episodes of healed plaque disruption or erosion. It has been demonstrated that an ongoing process of repeated arterial wound healing in human coronary arteries leads to a significant increase in plaque burden, progressing towards severe stenosis [24].

Progression of atherosclerotic plaque slowly leads to luminal narrowing and is generally asymptomatic until the plaque stenosis exceeds a certain percentage of the luminal diameter. These stenotic lesions could give rise to symptoms of clinically chronic stable CAD. All previously mentioned intact 'fibrous cap atheromas', isolating the thrombogenic fatty, necrotic core with a thick fibrous cap from the circulating blood, can lead to symptoms of stable CAD. Conversely, TCFA plaques are assumed to be more rupture-prone, frequently causing plaque ruptures and thereby leading to acute onsets of luminal superimposed thrombosis, resulting in the clinical manifestation of acute coronary syndrome (ACS) [20, 25, 26]. Plaque rupture is the most frequent underlying cause of coronary thrombus formation, followed by plaque erosion, typically characterized by an absent endothelium, minimal inflammation and abundant smooth muscle cells [25, 27]. Vulnerable plaques of the erosion-type are heterogenous and not clearly defined yet according to distinguishable plaque features [28]. In unstable angina pectoris or non-ST-segment elevation myocardial infarction, recurrent transient episodes of incomplete thrombotic vessel occlusion occur at either the site of plaque disruption or erosion. In acute ST-segment elevation myocardial infarction, an abrupt and persistent occluding thrombus in the infarct-related coronary artery is causing local cessation of myocardial perfusion, leading to myocardial necrosis.

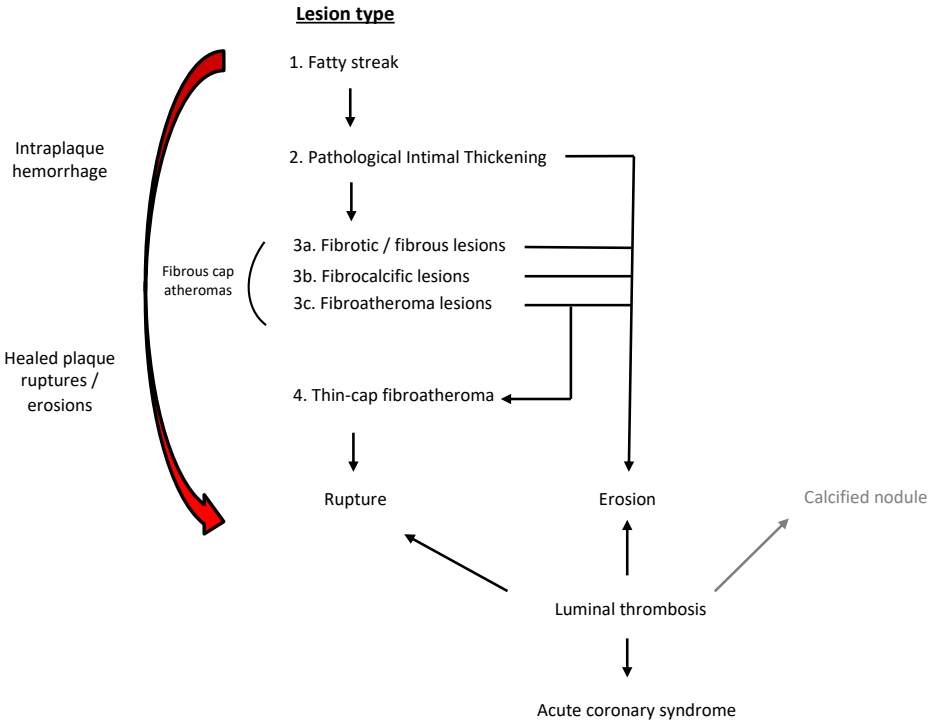


Figure 1. Simplified scheme of coronary atherosclerotic plaque progression.

Blood biomarkers & genetic polymorphisms: better understanding of human atherosclerosis, enhanced risk stratification and improved treatment benefit in established CAD

Discoveries in basic experimental research from laboratory and animal studies have been crucial in the development of understanding the underlying mechanisms in atherosclerosis. Despite considerable advances in learning the complex pathophysiology of atherosclerosis from these studies, significant results obtained in experiments may not be directly applied to humans [29]. For instance, this can be illustrated by the fact that large randomized controlled clinical trials were not able to demonstrate benefit of anti-oxidant supplements on CAD outcome [30]. Thus, we still lack definitive evidence to show that certain biological processes such as lipoprotein oxidation have a crucial causal role in human atherosclerosis [31]. Many questions in human CAD research have arisen and still remain unanswered. Furthermore, despite augmented knowledge, advanced diagnostic technologies and improved treatment strategies, atherosclerosis and its clinical sequelae continue to be an enormous disease burden globally. Hence, further clinical research within the field of underlying mechanisms in CAD

is indispensable.

In the meantime, in order to further improve cardiovascular outcome in CAD patients, risk stratification tools for purposes of secondary prevention should be further evaluated. Prognostication by identifying individuals at high risk of recurrent cardiovascular events remains a challenge. Nowadays, in clinical practice routinely used risk assessment tools for CAD consist mainly of determining conventional risk factors (e.g. Framingham risk score), integrated with clinical parameters from invasive (e.g. SYNTAX score) and non-invasive diagnostic tools (e.g. left ventricular function assessed by imaging) and well-established cardiovascular blood biomarkers (e.g. NT-proBNP, cardiac troponins). Advances have been made with several novel blood biomarkers, that independently of clinical factors carry prognostic information and improve risk stratification in established CAD [32]. By studying (novel) blood biomarkers in detail, not only valuable knowledge on atherosclerosis could be obtained in a non-invasive manner, but also the dynamic and versatile nature of atherosclerotic disease might be more accurately reflected.

In line with the usefulness of biomarkers, genetic information might be valuable as well in risk prediction. Common variants of genes involved in atherosclerosis, captured by genetic markers in the form of single-nucleotide polymorphisms (SNPs), might serve as risk predictors of CAD. After all, a positive family history significantly determines the risk of CAD, independently of traditional cardiovascular risk factors [33]. For example, carriers of a common genetic variant of haptoglobin (Hp2-2 genotype) were found out to have a 1.5-fold elevated risk for major cardiovascular events [23]. This suggests that genetic factors could potentially refine current CAD risk stratification [34]. Additionally, genetic variants involved in pharmacodynamic pathways could also be applied in predicting therapy response [35]. Thereby, treatment could be more targeted towards those who are most likely to benefit in order to enhance their likelihood of successful response. Thus, investigating and including genetic information has the potential to reach a more personalized and accurate risk stratification approach.

Outline of this thesis

Against the previously described pathophysiological background and remaining challenges within the field of atherosclerosis and CAD, the purpose of this thesis was three-fold: studying blood biomarkers for a better understanding of human atherosclerosis (1), enhanced risk stratification (2) and improved treatment benefit (3) in patients with established CAD. Therefore, different tools - which may reflect the patient's underlying coronary atherosclerotic disease - such as intracoronary imaging, (repeated) blood biomarker measurements and determination of certain genetic polymorphisms, have been investigated.

1. **Better understanding of human atherosclerosis by linking blood biomarkers to intracoronary imaging.**

   In the first part of this thesis, circulating blood biomarkers are studied in relation to coronary atherosclerosis by means of intracoronary imaging (virtual-histology intravascular ultrasound and near-infrared spectroscopy) in CAD patients. Investigating biological processes that might be linked to atherosclerosis increases our knowledge of the CAD pathogenesis and consequently may be useful for improving cardiovascular risk stratification or secondary prevention treatment strategies. Furthermore, the relationship between these biomarkers and 1-year cardiovascular outcome has been investigated as well to explore their potential additional value in cardiovascular risk prediction.

2. **Enhanced risk stratification in established CAD by studying blood biomarker patterns in detail.**

   In the second part, the behavioral temporal pattern after an acute coronary syndrome of novel and established biomarkers is described during 1-year follow-up. This has been performed by frequently repeated blood sample measurements, which gives us an unique insight in the value of these biomarkers for purposes of secondary cardiovascular risk prediction.

3. **Enhanced risk stratification and improved treatment benefit in established CAD by genetic polymorphisms.**

   In the third part, the relationship between some specific genetic polymorphisms and various cardiovascular outcome parameters has been studied in order to investigate its

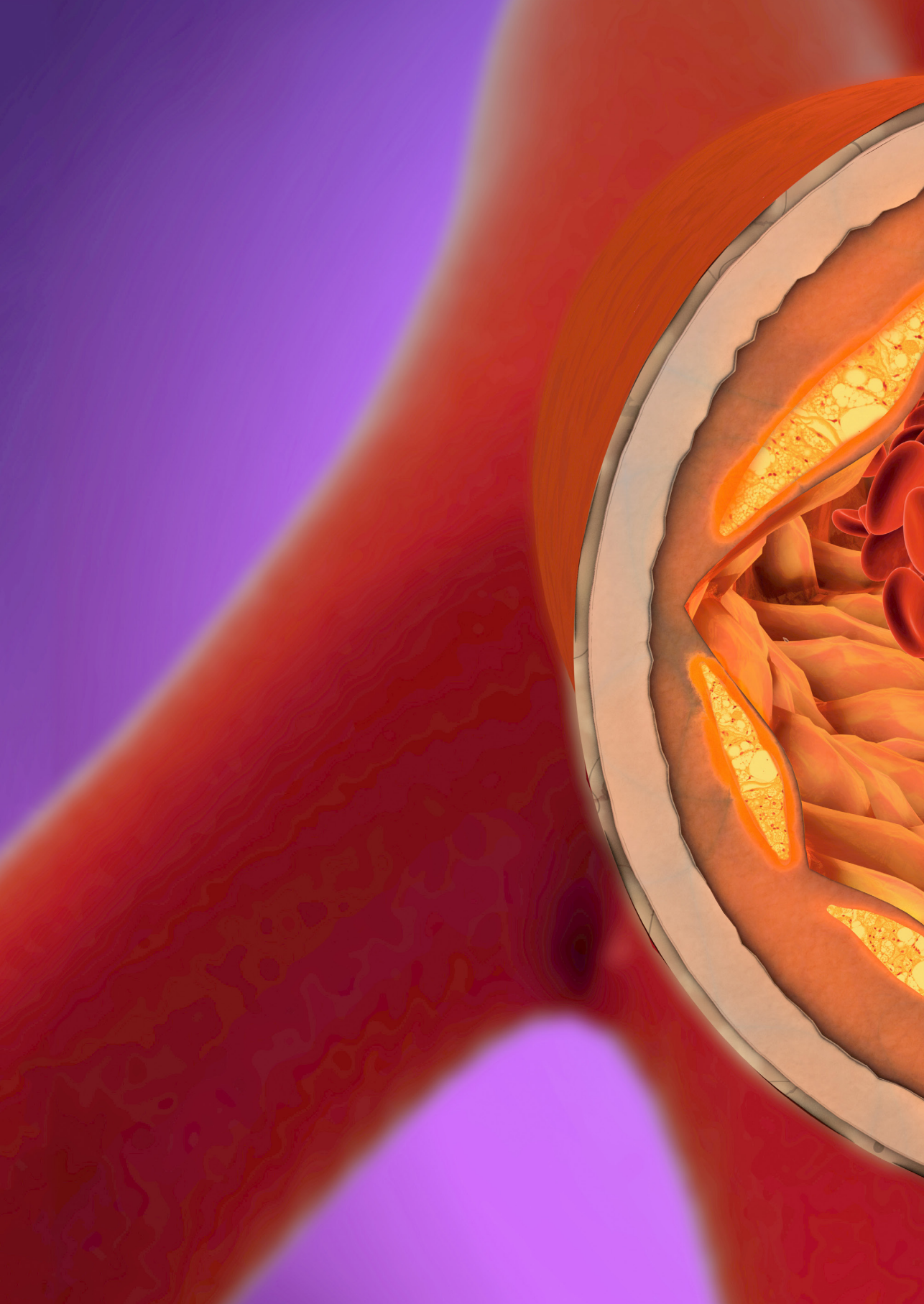
usefulness in cardiovascular risk stratification. Furthermore, the value of some genetic variants for targeting therapy in patients who would benefit most from ACE-inhibitors has been assessed as well.

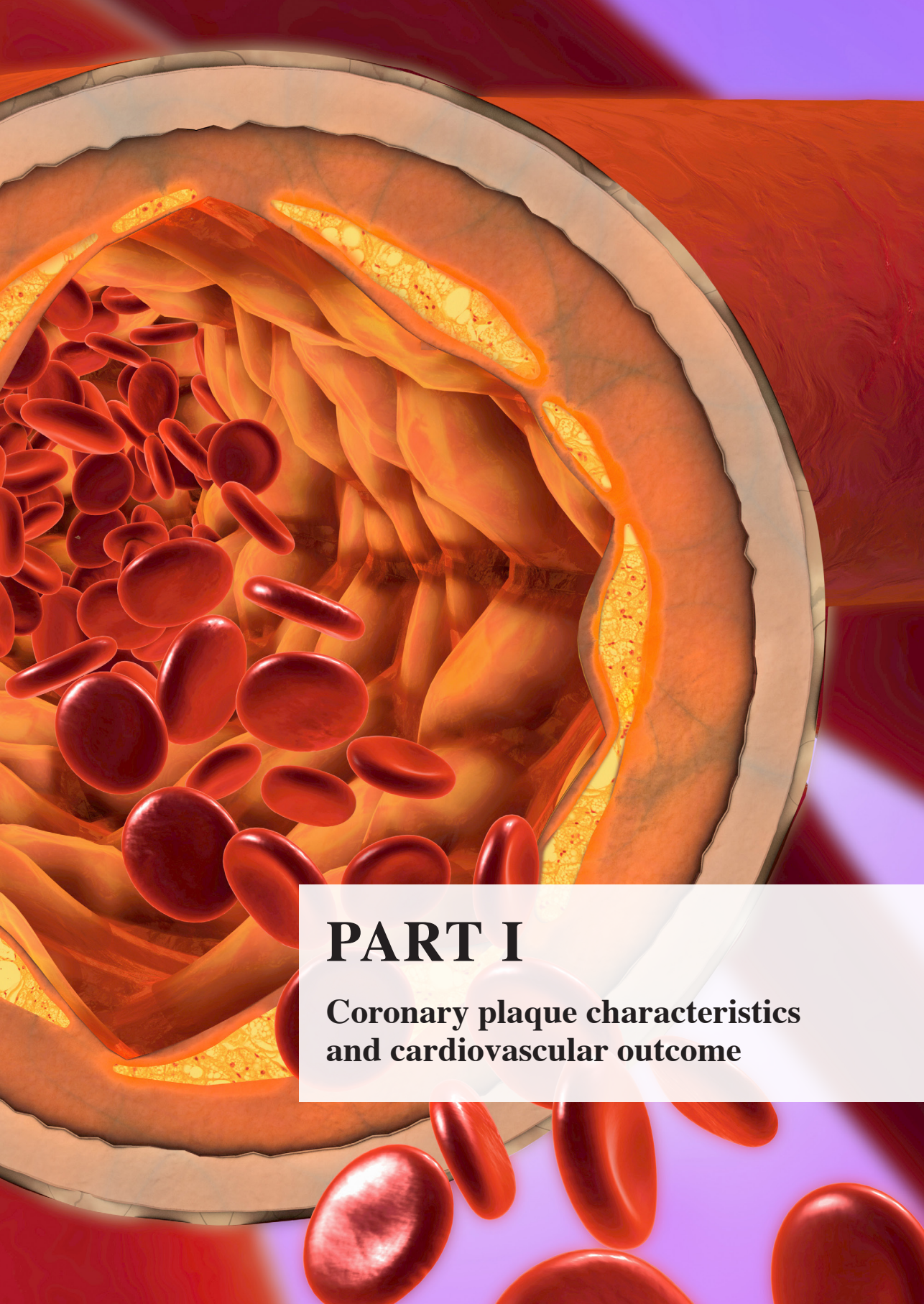
References

1. James S.L. et al. Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017. The Lancet 2018; 392: 1789-1858.
2. Roth G.A. et al. Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980–2017: a systematic analysis for the Global Burden of Disease Study 2017. The Lancet 2018; 392: 1736-1788.
3. Townsend N. et al. Cardiovascular disease in Europe: epidemiological update 2016. Eur Heart J 2016. 37: 3232-3245.
4. Benjamin E.J. et al. Heart Disease and Stroke Statistics-2018 Update: A Report From the American Heart Association. Circulation 2018; 137: e67-e492.
5. Barquera S. et al. Global Overview of the Epidemiology of Atherosclerotic Cardiovascular Disease. Arch Med Res 2015; 46: 328-338.
6. Okrainec K. et al. Coronary artery disease in the developing world. Am Heart J 2004; 148: 7-15.
7. Gimbrone M.A. Jr. and Garcia-Cardena G. Endothelial Cell Dysfunction and the Pathobiology of Atherosclerosis. Circ Res 2016; 118: 620-636.
8. Hansson G.K. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 2005; 352: 1685-1695.
9. Berliner J.A. et al. Atherosclerosis: basic mechanisms. Oxidation, inflammation, and genetics. Circulation 1995; 91: 2488-2496.
10. Young J.L. et al. Cytokines in the pathogenesis of atherosclerosis. Thromb Haemost 2002; 88: 554-567.
11. Douglas G. and Channon K.M. The pathogenesis of atherosclerosis. Medicine 2010; 38: 397-402.
12. Yusuf S. et al. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. Lancet 2004; 364: 937-952.
13. Ambrose J.A. and Barua R.S. The pathophysiology of cigarette smoking and cardiovascular disease: an update. J Am Coll Cardiol 2004; 43: 1731-1737.
14. Helkin A. et al. Dyslipidemia Part 1--Review of Lipid Metabolism and Vascular Cell Physiology. Vasc Endovascular Surg 2016; 50: 107-118.
15. Paneni F. et al. Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: part I. Eur Heart J 2013; 34: 2436-2443.
16. Touyz R.M. Reactive oxygen species, vascular oxidative stress, and redox signaling in hypertension: what is the clinical significance? Hypertension 2004; 44: 248-252.
17. Khera A.V. and Kathiresan S, Genetics of coronary artery disease: discovery, biology and clinical translation. Nat Rev Genet 2017; 18: 331-344.
18. Stary H.C. et al. A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation 1994; 89: 2462-2478.
19. Nakagawa K. and Nakashima Y. Pathologic intimal thickening in human atherosclerosis is formed by extracellular accumulation of plasma-derived lipids and dispersion of intimal smooth muscle cells. Atherosclerosis 2018; 274: 235-242.
20. Virmani R. et al. Lessons from sudden coronary death: a comprehensive morphological classification scheme

for atherosclerotic lesions. Arterioscler Thromb Vasc Biol 2000; 20: 1262-1275.

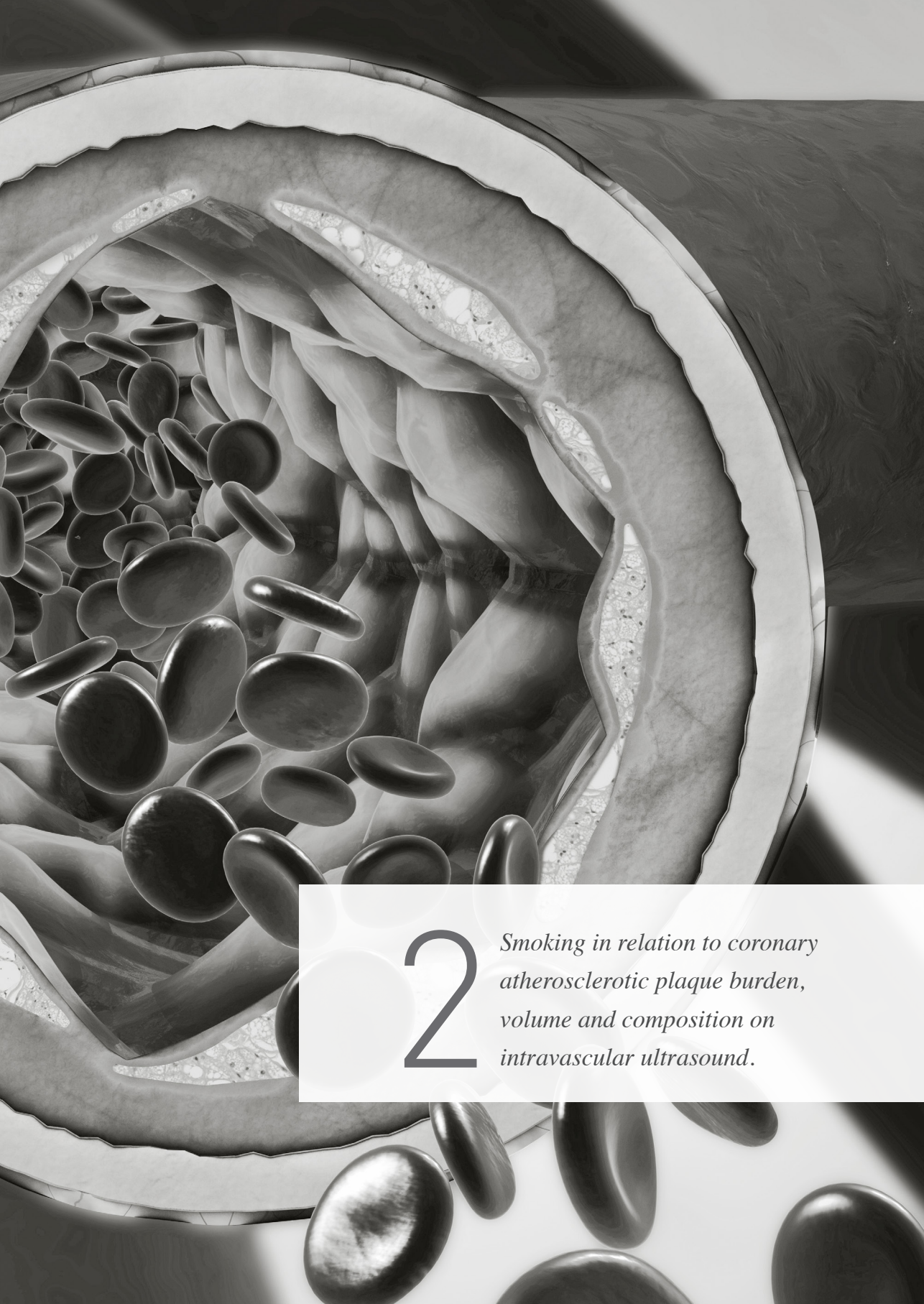
21. Kolodgie F.D. et al. Intraplaque hemorrhage and progression of coronary atheroma. N Engl J Med 2003; 349: 2316-2325.
22. Virmani R. et al. Atherosclerotic plaque progression and vulnerability to rupture: angiogenesis as a source of intraplaque hemorrhage. Arterioscler Thromb Vasc Biol 2005; 25: 2054-2061.
23. Viener H.L. and Levy A.P. Haptoglobin genotype and the iron hypothesis of atherosclerosis. Atherosclerosis 2011; 216: 17-18.
24. Burke A.P. et al. Healed plaque ruptures and sudden coronary death: evidence that subclinical rupture has a role in plaque progression. Circulation 2001; 103: 934-940.
25. Virmani R. et al. Pathology of the vulnerable plaque. J Am Coll Cardiol 2006; 47: C13-18.
26. Hong M.K. et al. Comparison of coronary plaque rupture between stable angina and acute myocardial infarction: a three-vessel intravascular ultrasound study in 235 patients. Circulation 2004; 110: 928-933.
27. Farb A. et al. Coronary plaque erosion without rupture into a lipid core. A frequent cause of coronary thrombosis in sudden coronary death. Circulation 1996; 93: 1354-1363.
28. Falk E. et al. Update on acute coronary syndromes: the pathologists' view. Eur Heart J 2013; 34: 719-728.
29. Libby P. et al. Progress and challenges in translating the biology of atherosclerosis. Nature 2011; 473: 317-325.
30. Ye Y. et al. Effect of antioxidant vitamin supplementation on cardiovascular outcomes: a meta-analysis of randomized controlled trials. PLoS One 2013; 8: e56803.
31. Steinberg D. and Witztum J.L. Is the oxidative modification hypothesis relevant to human atherosclerosis? Do the antioxidant trials conducted to date refute the hypothesis? Circulation 2002; 105: 2107-2111.
32. Omland T. and White H.D. State of the Art: Blood Biomarkers for Risk Stratification in Patients with Stable Ischemic Heart Disease. Clin Chem 2017; 63: 165-176.
33. Fischer M. et al. Distinct heritable patterns of angiographic coronary artery disease in families with myocardial infarction. Circulation 2005; 111: 855-862.
34. Bolton J.L. et al. Improvement in prediction of coronary heart disease risk over conventional risk factors using SNPs identified in genome-wide association studies. PLoS One 2013; 8: e57310.
35. Brugts J.J. et al. Genetic determinants of treatment benefit of the angiotensin-converting enzyme-inhibitor perindopril in patients with stable coronary artery disease. Eur Heart J 2010; 31: 1854-1864.





PART I

Coronary plaque characteristics and cardiovascular outcome



2

Smoking in relation to coronary atherosclerotic plaque burden, volume and composition on intravascular ultrasound.

2 Smoking in relation to coronary atherosclerotic plaque burden, volume and composition on intravascular ultrasound.

Buljubasic N, Akkerhuis KM, de Boer SP, Cheng JM, Garcia-Garcia HM, Lenzen MJ, Oemrawsingh RM, Battes LC, Rijndertse M, Regar E, Serruys PW, van Geuns RJ, Boersma E, Kardys I.

PLoS One. 2015; 10(10): e0141093. doi: 10.1371/journal.pone.0141093.

Abstract

Rationale: This study aimed to evaluate the relationship between cigarette smoking and coronary atherosclerotic burden, volume and composition as determined in-vivo by grayscale and virtual histology (VH) intravascular ultrasound (IVUS).

Methods & Results: Between 2008 and 2011, (VH-)IVUS of a non-culprit coronary artery was performed in 581 patients undergoing coronary angiography. To account for differences in baseline characteristics, current smokers were matched to never smokers by age, gender and indication for catheterization, resulting in 280 patients available for further analysis. Coronary atherosclerotic plaque volume, burden, composition (fibrous, fibro-fatty, dense calcium and necrotic core) and high-risk lesions (VH-IVUS derived thin-cap fibroatheroma (TCFA), plaque burden ≥70%, minimal luminal area ≤4.0 mm^2) were assessed. Cigarette smoking showed a tendency towards higher coronary plaque burden (mean±SD, 38.6±12.5% in current versus 36.4±11.0% in never smokers, p=0.080; and odds ratio (OR) of current smoking for plaque burden above versus below the median 1.69 (1.04 - 2.75), p=0.033). This effect was driven by an association in patients presenting with an acute coronary syndrome (ACS) (current smokers, plaque burden 38.3±12.8% versus never smokers, plaque burden

35.0±11.2%, p=0.049; OR 1.88 (1.02 - 3.44), p=0.042). Fibrous tissue tended to be lower in current smokers (mean±SD, 57.7±10.5% versus 60.4±12.6%, p=0.050) and fibro-fatty tissue was higher in current smokers (median [IQR], 9.6 [6.0 - 13.7]% versus 8.6 [5.8 - 12.2]%, p=0.039). However, differences in percentage necrotic core and dense calcium could not be demonstrated. Also, no differences were found with regard to high-risk lesions.

Conclusions: An association between smoking and degree of coronary atherosclerosis was present in patients undergoing coronary angiography who presented with ACS. Although smoking was associated with higher fibro-fatty percentage, no associations could be demonstrated with percentage necrotic core, nor with VH-IVUS derived TCFA lesions. Since the magnitude of the differences in both degree and composition of atherosclerosis was modest, clinical relevance of the findings may be questioned.

Introduction

Cigarette smoking is a well-known risk factor for developing coronary artery disease (CAD). Previous epidemiologic studies have demonstrated that cigarette smoking is associated with severity of atherosclerosis on both coronary angiography and coronary CT angiography [1,2], increased risk of myocardial infarction [3] and cardiovascular death [4,5].

In line with the above, several pathophysiologic effects of cigarette smoke exposure on cardiovascular function have been described. Both active and passive cigarette smoke exposure have been shown to promote endothelial dysfunction, stimulate inflammatory processes at the vessel wall and enhance vascular prothrombotic effects [6,7]. Thus, ample fundamental research evidence is available demonstrating that smoking directly impacts multiple aspects of atherosclerosis. However, less is currently known about the associations of smoking with in-vivo, macroscopic plaque composition and plaque vulnerability. Although coronary angiography enables evaluation of the unobstructed part of the lumen, it does not provide information on the structure of the arterial wall itself. Grayscale intravascular ultrasound (IVUS) also provides limited information on plaque characteristics.

Virtual histology (VH)-IVUS of the coronary arteries allows spectral analysis of backscattered radiofrequency ultrasound signal and herewith enables in-vivo analysis of the composition of atherosclerotic plaque as well as identification of thin-cap fibroatheroma

(TCFA) lesions [8]. Until now, the association between smoking and in-vivo coronary plaque composition has only been examined in two studies. The first [9,10] applied VH-IVUS and examined several plaque components, but did not assess VH-IVUS derived TCFA. The second [11] used integrated backscatter IVUS, which is based on the same principle as VH-IVUS, but examined 30 patients only.

The main objective of the current study is to evaluate the relationship between cigarette smoking and coronary atherosclerotic plaque burden, volume and composition as assessed by (VH-)IVUS, including VH-IVUS derived TCFA lesions, in patients undergoing coronary catheterization for stable coronary artery disease (CAD) or acute coronary syndrome (ACS). With this investigation we aim to improve our understanding of the complex pathophysiologic relation between cigarette smoke exposure and cardiovascular disease.

Methods

Study population and baseline characteristics

This study was performed within the framework of the European collaborative Project on Inflammation and Vascular Wall Remodeling in Atherosclerosis—Intravascular Ultrasound (ATHEROREMO-IVUS) study. The design of the ATHEROREMO-IVUS study has been described in detail elsewhere [12]. In brief, 581 patients who underwent diagnostic coronary angiography or percutaneous coronary intervention (PCI) for ACS or stable CAD have been included in this study between 2008 and 2011 at Erasmus MC, Rotterdam, the Netherlands. The ATHEROREMO-IVUS study has been approved by the human research ethics committee of the Erasmus MC. Written informed consent was obtained from all participants. The study is registered in ClinicalTrials.gov, number NCT01789411.

Baseline characteristics of the patients, including smoking status, were prospectively entered into a dedicated database. Smoking status was determined by self-report. Patients were categorized into those who currently smoke cigarettes (including those that had quit less than 1 year ago), those who had never smoked, and those who had smoked in the past (and had quit more than 1 year ago). For the current sub-study, patients from the full ATHEROREMO-IVUS study cohort were eligible when they were current or never smokers. Patients who had quit smoking more than 1 year ago (n=104), or for whom information on smoking was lacking (n=1), were excluded, leaving 476 patients eligible for analysis.

Intravascular ultrasound

Following the standard coronary angiography or PCI procedure, IVUS imaging of a non-culprit coronary artery was performed. The predefined order of preference for selection of the non-culprit vessel was: 1. left anterior descending (LAD) artery; 2. right coronary artery (RCA); 3. left circumflex (LCX) artery. All IVUS data were acquired with the Volcano™ s5/s5i Imaging System (Volcano Corp., San Diego, CA, USA), using a Volcano™ Eagle Eye™ Gold IVUS catheter (20 MHz). An automatic pullback system was used with a standard pull back speed of 0.5 mm per second. IVUS images were analyzed offline by an independent core laboratory (Cardialysis BV, Rotterdam, the Netherlands) that had no knowledge of clinical data. IVUS grayscale and virtual histology analyses were performed using pcVH 2.1 and qVH (Volcano Corp., San Diego, CA, USA) software.

The external elastic membrane and luminal borders were contoured for each frame (median interslice distance, 0.40 mm). Degree and phenotype of the atherosclerotic plaque were assessed. Plaque volume was defined as the percent of the volume of the external elastic membrane occupied by atheroma, i.e. percent atheroma volume. Plaque burden was defined as plaque and media cross-sectional area divided by external elastic membrane cross-sectional area. A coronary lesion was defined as a segment with a plaque burden of more than 40% in at least 3 consecutive frames. Using VH-IVUS, the composition of the atherosclerotic plaques was characterized into 4 different tissue types: fibrous (FI), fibro-fatty (FF), dense calcium (DC) and necrotic core (NC) [13]. These tissue type components were expressed as percentages of total plaque volume. Three types of high-risk lesions were identified: 1. VH-IVUS derived thin-cap fibroatheroma (TCFA) lesion, defined as a lesion with presence of >10% confluent necrotic core in direct contact with the lumen; 2. Lesion with large plaque burden, defined as a lesion with a plaque burden of ≥70%; 3. Stenotic lesion, defined as a lesion with a minimal luminal area of ≤4.0 mm^2 [13,14]. In addition, remodeling index was calculated and expressed as the external elastic membrane cross-sectional area at the site of minimal luminal area divided by the reference external elastic membrane cross-sectional area. The reference site was selected <10 mm proximal to the lesion. Positive remodeling (arterial expansion) was defined as a remodeling index of >1.05, and negative remodeling (arterial shrinkage) was defined as a remodeling index of <0.95.

Statistical analysis

Categorical data are presented as numbers and percentages. Normality of the distributions

of continuous variables was examined by visual inspection of the histogram and by normal Q-Q plots. Continuous data are presented as mean±standard deviation (SD) or as median and interquartile range (IQR), depending on their distribution. Plaque volume, percentage fibro-fatty volume (% FF) and percentage dense calcium volume (% DC) appeared to be non-normally distributed and were therefore ln-transformed for further analyses.

Baseline clinical and procedural characteristics of current smokers and those who had never smoked were compared using the independent Student's t-test for continuous variables and using the χ^2 test for categorical variables. Subsequently, to account for differences in baseline characteristics between current smokers and those who had never smoked, we performed a matching procedure. Every current smoker was matched to a never smoker by age (±5 years), gender and indication for catheterization (acute coronary syndrome or stable angina pectoris).

In the matched set, baseline clinical, procedural and (VH-)IVUS characteristics of current smokers and never smokers were compared using the paired samples t-test for continuous variables and the McNemar test or marginal homogeneity test for categorical variables, whichever was appropriate.

Subsequently, we performed conditional logistic regression to examine the associations between smoking status and high plaque burden (above versus below the median), as well as smoking status and the three types of high-risk lesions (VH-IVUS derived TCFA, lesion with plaque burden ≥70%, lesion with minimal luminal area ≤4.0 mm^2).

Finally, to examine effect modification by age and indication for catheterization, we stratified on these variables and repeated all the above described analyses in subgroups. For this purpose, we divided age into tertiles (based on age of the smokers). All data were analyzed with SPSS software (SPSS 20.0, IBM corp., Armonk, NY, USA). All statistical tests were two-tailed and p-values <0.050 were considered statistically significant.

Results

Baseline characteristics

Baseline clinical and procedural characteristics of the total patient population are presented in Table 1. Current (n=169) and never smokers (n=307) differed significantly at baseline. Current smokers, on average, were significantly younger (55.7±10.8 years vs. 64.4±10.8, p<0.001) than

the never smokers. Significantly more men were present among the current smokers (79.3% vs. 70.7%, p=0.041), and current smokers were less likely to have predisposing risk factors such as hypertension (p<0.001), dyslipidemia (p=0.030) and diabetes mellitus (p=0.038). Furthermore, the indication for coronary angiography or PCI also differed significantly between the two groups. Current smokers more often underwent catheterization for ACS and less often for stable CAD compared to the never smokers (p<0.001).

After the matching procedure, baseline clinical and procedural characteristics were similarly distributed between the two groups (Table 2).

Table 1. Baseline clinical and procedural characteristics, before matching.

	Current smokers (n = 169)	Never smokers (n = 307)	P-value
Patient characteristics			
Age, years	55.7 ± 10.8	64.4 ± 10.8	<0.001
Male gender, n (%)	134 (79.3)	217 (70.7)	0.041
Hypertension, n (%)	63 (37.5)	171 (55.7)	<0.001
Dyslipidemia, n (%)	80 (47.6)	178 (58.0)	0.030
Diabetes mellitus, n (%)	20 (11.8)	59 (19.2)	0.038
Positive family history, n (%)	86 (51.2)	158 (51.5)	0.95
Peripheral artery disease, n (%)	12 (7.1)	15 (4.9)	0.32
Previous MI, n (%)	38 (22.5)	103 (33.6)	0.011
Previous PCI, n (%)	37 (21.9)	103 (33.6)	0.008
Previous CABG, n (%)	1 (0.6)	12 (3.9)	0.034
Previous stroke, n (%)	4 (2.4)	16 (5.2)	0.14
History of renal insufficiency, n (%)	8 (4.7)	17 (5.5)	0.71
Procedural characteristics			
Indication for catheterization			<0.001
Acute coronary syndrome, n (%)	119 (72.1)	151 (49.5)	
Stable angina pectoris, n (%)	46 (27.9)	154 (50.5)	
Coronary artery disease			0.24
No significant stenosis, n (%)	9 (5.3)	27 (8.8)	
1-vessel disease, n (%)	90 (53.3)	151 (49.2)	
2-vessel disease, n (%)	56 (33.1)	91 (29.6)	
3-vessel disease, n (%)	14 (8.3)	38 (12.4)	
Vessel imaged by VH-IVUS			0.15
LAD , n (%)	71 (42.0)	101 (33.1)	
RCA , n (%)	44 (26.0)	95 (31.1)	
LCX , n (%)	54 (32.0)	109 (35.7)	

CABG=coronary artery bypass grafting; LAD=left anterior descending artery; LCX=left circumflex artery; MI=myocardial infarction; PCI=percutaneous coronary intervention; RCA=right coronary artery. Values are mean ± SD or n (%). P-values were obtained by independent samples t-test or Chi-squared test, whichever was appropriate.

Table 2. Baseline clinical and procedural characteristics, after matching.

	Current smokers (n = 140)	Never smokers (n = 140)	P-value
Patient characteristics			
Age, years	57.9 ± 9.7	58.1 ± 9.5	MV
Male gender, n (%)	108 (77.1)	108 (77.1)	MV
Hypertension, n (%)	58 (41.4)	73 (52.1)	0.10
Dyslipidemia, n (%)	74 (52.9)	74 (52.9)	1.00
Diabetes mellitus, n (%)	19 (13.6)	28 (20.0)	0.21
Positive family history, n (%)	68 (48.9)	75 (53.6)	0.53
Peripheral artery disease, n (%)	12 (8.6)	7 (5.0)	0.36
Previous MI, n (%)	33 (23.6)	35 (25.0)	0.89
Previous PCI, n (%)	33 (23.6)	45 (32.1)	0.11
Previous CABG, n (%)	0 (0.0)	3 (2.1)	0.25
Previous stroke, n (%)	4 (2.9)	7 (5.0)	0.51
History of renal insufficiency, n (%)	6 (4.3)	11 (7.9)	0.33
Procedural characteristics			
Indication for catheterization			MV
Acute coronary syndrome, n (%)	96 (68.6)	96 (68.6)	
Stable angina pectoris, n (%)	44 (31.4)	44 (31.4)	
Coronary artery disease			0.33
No significant stenosis, n (%)	6 (4.3)	14 (10.0)	
1-vessel disease, n (%)	74 (52.9)	76 (54.3)	
2-vessel disease, n (%)	47 (33.6)	36 (25.7)	
3-vessel disease, n (%)	13 (9.3)	14 (10.0)	
Vessel imaged by VH-IVUS			0.51
LAD , n (%)	56 (40.0)	50 (35.7)	
RCA , n (%)	40 (28.6)	43 (30.7)	
LCX , n (%)	44 (31.4)	47 (33.6)	

CABG=coronary artery bypass grafting; LAD=left anterior descending artery; LCX=left circumflex artery; MI=myocardial infarction; MV=matching variable; PCI=percutaneous coronary intervention; RCA=right coronary artery. Values are mean ± SD or n (%). P-values were obtained by paired samples t-test, McNemar test or Marginal Homogeneity, whichever was appropriate.

Degree of coronary atherosclerosis

To assess differences in degree of atherosclerosis between current smokers and never smokers, plaque volume and plaque burden were examined in the coronary segments. Plaque volume (median [IQR]) was similar for current and never smokers (221.8 [134.6 - 312.5]mm^3 versus 207.5 [134.5 - 293.2]mm^3) (Table 3). On the other hand, with regard to plaque burden, there was a tendency towards higher values in current smokers (Table 3). Plaque burden (mean±SD) was 38.6±12.5% in current smokers versus 36.4±11.0% in never smokers, p=0.080 (Figure 1).

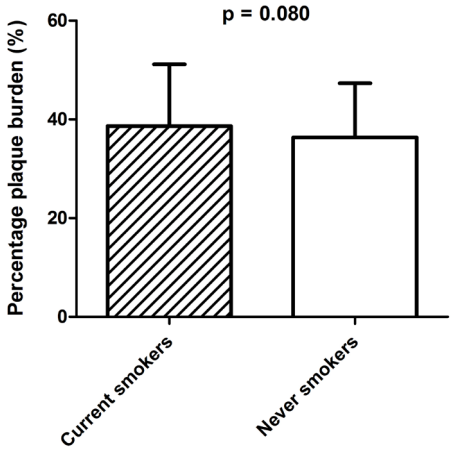


Figure 1. Difference in plaque burden between current and never smokers.

The odds ratio (OR) (95% confidence interval (CI)) of current smoking for plaque burden above the median versus below the median was 1.69 (1.04 - 2.75), p=0.033 (Table 4). After stratification on age, this tendency towards higher plaque burden in current smokers was only present in the lower age tertile (37.8±12.6% versus 33.9±11.1%, p=0.09). However, the OR for plaque burden above the median versus below the median was not significant in this subgroup (Supplementary Tables 1 and 2). Furthermore, after stratification on indication for catheterization, plaque burden was significantly higher in current smokers presenting with ACS (38.3±12.8% versus 35.0±11.2%, p=0.049 and OR 1.88 (1.02 - 3.44), p=0.042) (Supplementary Tables 3 and 4).

The number of patients with ≥1 lesions was similar in current and never smokers (85.7% vs. 87.9%, p=0.72) (Table 3). The odds ratio of having one or more lesions with plaque burden ≥70% was also similar (OR (95% CI): 1.47 (0.76 - 2.83)), as was the odds ratio of having one or more lesions with a minimal luminal area of ≤4.0 mm^2 (Table 4). Subgroup analysis did not provide additional insights.

As described above, we found a borderline association with plaque burden, but no association with plaque volume. This seeming discrepancy may be due to the fact that plaque burden is not a direct measure of three dimensional plaque volume, but rather a two dimensional measure that also accounts for arterial wall remodeling. Specifically, the discrepancy may be explained by an association with negative remodeling. Therefore, we examined associations of smoking with remodeling in a post-hoc analysis. Smoking displayed a tendency toward

a positive association with negative remodeling (OR (95% CI): 1.58 (0.89 - 2.81), p=0.12), as well as a tendency toward a negative association with positive remodeling (OR (95% CI): 0.47 (0.21 - 1.05), p=0.065).

Table 3. (VH-)IVUS segment and lesion characteristics, after matching.

	Current smokers (n = 140)	Never smokers (n = 140)	P-value
(VH-)IVUS segment parameters			
Segment length, mm	45.4 ± 15.4	44.7 ± 13.2	0.67
Degree of atherosclerosis			
Plaque volume, mm^3	221.8 [134.6 - 312.5]	207.5 [134.5 - 293.2]	0.60
Plaque burden, %	38.6 ± 12.5	36.4 ± 11.0	0.080
Composition of atherosclerosis			
% FI volume	57.7 ± 10.5	60.4 ± 12.6	0.050
% FF volume	9.6 [6.0 - 13.7]	8.6 [5.8 - 12.2]	0.039
% NC volume	21.6 ± 8.0	20.8 ± 8.8	0.37
% DC volume	7.6 [4.7 - 13.9]	8.0 [4.3 - 13.3]	0.62
(VH-)IVUS lesion parameters			
≥1 Lesions, n (%)	120 (85.7)	123 (87.9)	0.73
Presence of high risk lesions, n (%)	91 (64.3)	83 (59.3)	0.46
High risk lesion type:			
Degree of atherosclerosis			
≥1 Lesion with plaque burden ≥70%, n (%)	32 (22.1)	27 (19.3)	0.65
≥1 Lesion with MLA ≤4.0mm^2, n (%)	43 (30.7)	42 (30.0)	1.00
Composition of atherosclerosis			
≥1 TCFA, n (%)	57 (40.7)	57 (40.7)	1.00

FI=fibrous; FF=fibro-fatty; NC=necrotic core; DC=dense calcium; MLA=minimal lumen area; TCFA=thin-cap fibroatheroma. Values are mean ± SD, median [interquartile range], or n (%). P-values were obtained by paired samples t-test or McNemar test, whichever was appropriate.

Table 4. Odds ratios of current smoking for high plaque burden and for presence of high risk lesion types.

	OR (95% CI)	P-value
(VH-)IVUS segment parameters		
Plaque burden		
Below the median	1.00 (reference)	
Above the median	1.69 (1.04 - 2.75)	0.033
(VH-)IVUS lesion parameters		
≥1 Lesion with plaque burden ≥70%	1.20 (0.66 - 2.17)	0.55
≥1 Lesion with MLA ≤4.0mm^2	1.03 (0.63 - 1.71)	0.90
≥1 TCFA	1.00 (0.63 - 1.60)	1.00

OR=odds ratio; CI=confidence interval; MLA=minimal lumen area; TCFA=thin-cap fibroatheroma. P-values were obtained by conditional logistic regression.

Composition of coronary atherosclerosis

VH-IVUS segment and lesion characteristics of the matched smokers and never smokers are listed in Table 3. Percentage of fibrous tissue (% FI) volume in the examined coronary segment tended to be lower in current smokers (57.7±10.5% vs. 60.4±12.6%, p=0.050), which was driven by the lower age tertile (56.5±10.4% vs. 61.4±12.8%, p=0.042) (Supplementary Table 1). Percentage of fibro-fatty tissue volume was higher in current smokers (9.6 [6.0 - 13.7]% vs. 8.6 [5.8 - 12.2]%, p=0.039) (Table 3 and Figure 2), which was driven by the upper age tertile (11.1 [6.3 - 15.3]% vs. 8.3 [5.8 - 12.3], p=0.08) (Supplementary Table 1). However, differences in percentage necrotic core (% NC) volume and dense calcium volume could not be demonstrated, and prevalence of ≥1 TCFA lesions was the same in current and never smokers (both 40.7%, Tables 3 and 4). After stratification on age, TCFA lesions tended to occur less often in current smokers in the upper age tertile (23.4% vs 44.7%, p=0.06; OR 0.41 (0.17 - 0.99, p=0.048) (Supplementary Tables 1 and 2).

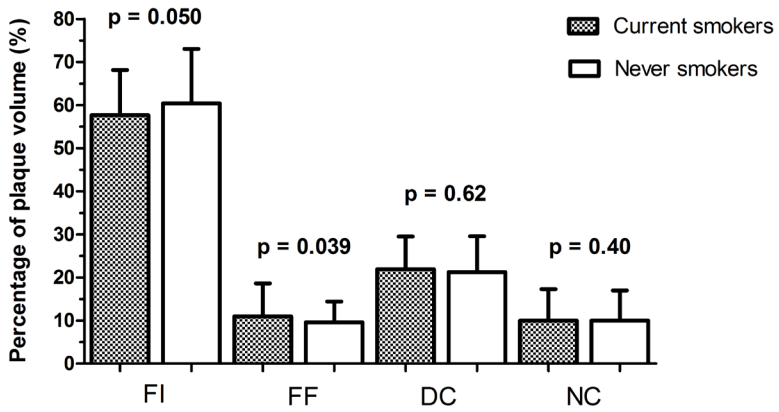


Figure 2. Difference in composition of coronary atherosclerosis between current and never smokers, after matching.

FI=fibrous; FF=fibro-fatty; DC=dense calcium; NC=necrotic core.

Discussion

This study investigated the associations of cigarette smoking with coronary atherosclerotic plaque burden, volume and composition as determined by (VH-)IVUS of a non-culprit section of a coronary artery in patients undergoing coronary angiography. Cigarette smoking showed a tendency towards higher coronary plaque burden, which was driven by an association in the subgroup of patients presenting with ACS. The magnitude of this effect was very modest. Furthermore, while smoking was associated with higher percentage of fibro-fatty plaque volume, no associations could be demonstrated with percentage necrotic core volume, nor with VH-IVUS derived TCFA lesions, suggesting that smoking has no major influence on plaque vulnerability.

Although several studies have examined the association between smoking and degree of coronary atherosclerosis as measured by IVUS [9–11,15–17], so far only one large study has applied virtual histology (VH-IVUS) to assess its association with composition of coronary atherosclerosis and plaque vulnerability. Philipp et al [10] found that smoking was not associated with plaque composition in a sample of 990 consecutive, non-selected patients, which is in line with our findings. However, they did not perform a lesion-based analysis, or a categorization into high-risk lesions such as TCFA, as we did. Missel et al [9] examined a subset of 473 male patients with de novo culprit coronary lesions from the same registry, and found a higher NC/DC ratio, a measure of plaque vulnerability, in smokers. Other VH parameters were not significantly influenced by smoking. A post-hoc analysis in our dataset showed no relation between smoking and NC/DC ratio (results not shown). In a small, underpowered study of 30 patients with stable angina Sano et al [11] found no association of plaque characteristics with smoking either. Remarkably, in our subgroup analysis, we found that TCFA lesions tended to occur less often in current smokers in the upper age tertile. A healthy survivor effect may pose a potential explanation for this finding.

With regard to degree of coronary atherosclerosis as assessed by IVUS, previous studies have rendered contradicting results. Nicholls et al [16] demonstrated that smoking was a weak independent predictor of percent plaque volume in 654 patients with a clinical indication for diagnostic coronary angiography. Furthermore, Von Birgelen et al [17] found an association between smoking and progression of plaque plus media cross-sectional area in 56 patients with de novo, hemodynamically nonsignificant plaques. In contrast, Kahlon et al concluded that smoking was not correlated with plaque burden in 897 consecutive patients undergoing

IVUS investigation [15]. In our total study population, the association between smoking and plaque burden as well as plaque volume was not substantial. However, we did find such an association in the subgroup of patients presenting with ACS. In this subgroup, current smokers showed a higher plaque burden than never smokers. These findings concur with the fact that smoking is associated with both greater degrees of stenosis and an increased likelihood of acute plaque events [18], as well as with the fact that smoking is associated with reduced fibrinolytic potential and thus a pro-thrombotic phenotype [18]. Nevertheless, it should be recognized that the magnitude of the difference in degree of atherosclerosis between current and never smokers presenting with ACS was modest in our study, and that both pathophysiologic and clinical relevance of the findings may be questioned. Previous studies on smoking and degree of atherosclerosis on IVUS have not stratified their results on indication for angiography.

Our results do not support the hypothesis that smoking is associated with coronary plaque vulnerability. This may be explained by the possibility that plaque erosion, and not as much vulnerable plaque rupture, is the intermediate between smoking and cardiac adverse events. Histopathological studies have shown that luminal thrombosis may result from two different pathologies, namely plaque rupture and plaque erosion [19–22]. Plaque rupture seems to be highly associated with TCFAs and causes thrombotic coronary occlusion. Plaque erosion is characterized by an acute thrombus in direct contact with the intimal plaque, rich in smooth muscle cells with surrounding proteoglycan matrix and minimal inflammation. The lesions tend to be eccentric, infrequently calcified and cause less severe narrowing at sites of thrombosis [20,21]. Most eroded lesions have an absent or poorly defined necrotic core, which, when present, is not in close proximity to the luminal thrombus. Studies have shown that smoking is associated with plaque erosion and frequently causes coronary thrombosis [20,21]. This may possibly explain the general absence of an association of smoking with coronary plaque composition as assessed by VH-IVUS in the literature, and the inconsistent findings with regard to smoking and degree of coronary atherosclerosis as assessed by grayscale IVUS. We found that current smokers tend to have a slightly lower percentage fibrous plaque volume (driven by the lower age tertile), and that they have a somewhat higher percentage fibro-fatty plaque volume; however, this trend did not persist with regard to percentage necrotic core or presence of TCFA. These findings do not preclude plaque erosion as the underlying mechanism. Moreover, histopathological studies examining coronary arteries suggest that smoking predisposes patients to coronary thrombosis rather than promoting

the progression of atherosclerosis [21,23,24]. These findings are supported by clinical patient studies showing that smokers seem to have a more favourable response to fibrinolytic therapy compared to nonsmokers, which may be attributed to their hypercoagulable state [25–27]. In the present study, we did not focus on the influence of smoking on blood coagulation.

Smoking displayed a tendency toward a positive association with negative remodeling, as well as a tendency toward a negative association with positive remodeling. A possible explanation for these seemingly counterintuitive findings lies in the interpretation of the early phases of remodeling. Modest positive lesion remodeling may be considered as a physiological, and thus favourable, response to progression of atherosclerotic plaque (also known as the Glagov adaptive phenomenon) [28]. In this light, smoking may point towards a lower adaptive capacity to atherosclerotic burden.

Some aspects of this study warrant consideration. A single non-culprit coronary vessel was imaged. This study design of ATHEROREMO-IVUS was based on the hypothesis that such a non-stenotic segment adequately reflects the state of the coronary wall of the larger coronary tree [12]. Both ex vivo and in vivo studies using IVUS in patients presenting with myocardial infarction have demonstrated the existence of additional TCFAs other than the culprit lesion in the culprit artery, as well as TCFAs in other arteries than the culprit artery [29]. Accordingly, the results of ATHEROREMO-IVUS, which we published earlier, have confirmed that the characteristics of the coronary wall of this non-culprit coronary vessel are strongly associated with subsequent cardiovascular outcome [30]. In addition, previous studies evaluating IVUS have similarly demonstrated that the coronary wall of comparable non-culprit, non-stenotic segments of a single vessel does reflect larger coronary disease burden and is associated with subsequent events [31,32]. Nevertheless, simultaneous assessment of the culprit vessel might have provided additional insights into the underlying disease mechanisms. Another limitation of this study is that IVUS is formally not capable of detecting the TCFA according to histopathological definitions [33,34]. Nonetheless, a concept of VH-IVUS derived TCFA has been postulated for plaques with a plaque burden ≥40% and a confluent necrotic core ≥10% in direct contact with the lumen in at least three VH-IVUS frames [14,33], and we have demonstrated earlier that such VH-IVUS derived TCFA lesions strongly and independently predict the occurrence of major adverse cardiac events within the current study population [30]. Furthermore, smoking status was determined by self-report in this cross-sectional study. To minimize the risk of misclassification, we excluded former smokers from our study. Finally, a matching procedure was necessary because of differences

in baseline characteristics between smokers and never smokers. Since part of the smokers (n=29) could not be matched to a never smoker, this study design entailed some loss of statistical power. Moreover, statistical power for the stratified analyses was limited.

In conclusion, we were not able to demonstrate a clear and strong association of cigarette smoking with degree of atherosclerosis and coronary plaque vulnerability as assessed by VH-IVUS in the current study. Additional studies, using various intravascular imaging modalities, are needed to further describe the association between smoking and in-vivo degree and composition of coronary plaque, and to herewith discern the mechanisms underlying the association between smoking and cardiac adverse events.

References

1. Kim J.A. et al. Relationship between amount of cigarette smoking and coronary atherosclerosis on coronary CTA in asymptomatic individuals. Int J Cardiovasc Imaging 2013; 29: 21–28.
2. Waters D. et al. Effects of cigarette smoking on the angiographic evolution of coronary atherosclerosis. A Canadian Coronary Atherosclerosis Intervention Trial (CCAIT) Substudy. CCAIT Study Group. Circulation 1996; 94: 614–621.
3. Njolstad I. et al. Smoking, serum lipids, blood pressure, and sex differences in myocardial infarction. A 12-year follow-up of the Finnmark Study. Circulation 1996; 93: 450–456.
4. Escobedo L.G. and Zack M.M. Comparison of sudden and nonsudden coronary deaths in the United States. Circulation 1996; 93: 2033–2036.
5. Lakier J.B. Smoking and cardiovascular disease. Am J Med 1992; 93: 8S–12S.
6. Ambrose J.A. and Barua R.S. The pathophysiology of cigarette smoking and cardiovascular disease: an update. J Am Coll Cardiol 2004; 43: 1731–1737.
7. Benowitz N.L. Cigarette smoking and cardiovascular disease: pathophysiology and implications for treatment. Prog Cardiovasc Dis 2003; 46: 91–111.
8. Stone G.W. et al. A prospective natural history study of coronary atherosclerosis. N Engl J Med 2011; 364: 226–235.
9. Missel E. et al. In vivo virtual histology intravascular ultrasound correlates of risk factors for sudden coronary death in men: results from the prospective, multi-centre virtual histology intravascular ultrasound registry. Eur Heart J 2008; 29: 2141–2147.
10. Philipp S. et al. Do systemic risk factors impact invasive findings from virtual histology? Insights from the international virtual histology registry. Eur Heart J 2010; 31: 196–202.
11. Sano K. et al. In vivo quantitative tissue characterization of angiographically normal coronary lesions and the relation with risk factors: a study using integrated backscatter intravascular ultrasound. Circ J 2005; 69: 543–549.
12. de Boer S.P. et al. Relation of genetic profile and novel circulating biomarkers with coronary plaque phenotype as determined by intravascular ultrasound: rationale and design of the ATHEROREMO-IVUS study. EuroIntervention 2014; 10: 953–960.
13. Garcia-Garcia H.M. et al. Tissue characterization using intravascular radiofrequency data analysis: recommendations for acquisition, analysis, interpretation and reporting. EuroIntervention 2009; 5: 177–189.
14. Rodriguez-Granillo G.A. et al. In vivo intravascular ultrasound-derived thin-cap fibroatheroma detection using ultrasound radiofrequency data analysis. J Am Coll Cardiol 2005; 46: 2038–2042.
15. Kahlon J.P. et al. The impact of coronary artery disease risk factors on intravascular ultrasound-derived morphologic indices of human coronaries. Echocardiography 2006; 23: 308–311.
16. Nicholls S.J. et al. Relationship between cardiovascular risk factors and atherosclerotic disease burden measured by intravascular ultrasound. J Am Coll Cardiol 2006; 47: 1967–1975.
17. von Birgelen C. et al, Relationship between cardiovascular risk as predicted by established risk scores versus plaque progression as measured by serial intravascular ultrasound in left main coronary arteries. Circulation 2004; 110: 1579–1585.
18. Newby D.E. Triggering of acute myocardial infarction: beyond the vulnerable plaque. Heart 2010; 96: 1247–1251.

19. Virmani R. et al. Pathology of the vulnerable plaque. J Am Coll Cardiol 2006; 47: C13–18.
20. Farb A. et al. Coronary plaque erosion without rupture into a lipid core. A frequent cause of coronary thrombosis in sudden coronary death. Circulation 1996; 93: 1354–1363.
21. Burke A.P. et al. Effect of risk factors on the mechanism of acute thrombosis and sudden coronary death in women. Circulation 1998; 97: 2110–2116.
22. Burke A.P. et al. Atherosclerotic plaque morphology and coronary thrombi. J Nucl Cardiol 2002; 9: 95–103.
23. Zieske A.W. et al. Smoking is associated with advanced coronary atherosclerosis in youth. Atherosclerosis 2005; 180: 87–92.
24. Burke A.P. et al. Coronary risk factors and plaque morphology in men with coronary disease who died suddenly. N Engl J Med 1997; 336: 1276–1282.
25. Grines C.L. et al. Effect of cigarette smoking on outcome after thrombolytic therapy for myocardial infarction. Circulation 1995; 91: 298–303.
26. Newby D.E. et al. Impaired coronary tissue plasminogen activator release is associated with coronary atherosclerosis and cigarette smoking: direct link between endothelial dysfunction and atherothrombosis. Circulation 2001; 103: 1936–1941.
27. Barua R.S. and Ambrose J.A. Mechanisms of coronary thrombosis in cigarette smoke exposure. Arterioscler Thromb Vasc Biol 2013; 33: 1460–1467.
28. Korshunov V.A. et al. Vascular remodeling: hemodynamic and biochemical mechanisms underlying Glagov's phenomenon. Arterioscler Thromb Vasc Biol 2007; 27: 1722–1728.
29. Libby P. Atherosclerosis: disease biology affecting the coronary vasculature. Am J Cardiol 2006; 98: 3Q–9Q.
30. Cheng J.M. et al. In vivo detection of high-risk coronary plaques by radiofrequency intravascular ultrasound and cardiovascular outcome: results of the ATHEROREMO-IVUS study. Eur Heart J 2014; 35: 639–647.
31. Nicholls S.J. et al. Intravascular ultrasound-derived measures of coronary atherosclerotic plaque burden and clinical outcome. J Am Coll Cardiol 2010; 55: 2399–2407.
32. Puri R. et al. Coronary atheroma volume and cardiovascular events during maximally intensive statin therapy. Eur Heart J 2013; 34: 3182–3190.
33. Garcia-Garcia H.M. et al. Imaging of coronary atherosclerosis: intravascular ultrasound. Eur Heart J 2010; 31: 2456–2469.
34. Virmani R. Are our tools for the identification of TCFA ready and do we know them? JACC Cardiovasc imaging 2011; 4: 656–658.

Supplementary Table 1. (VH-)IVUS segment and lesion characteristics in the matched set, stratified on age.

	Lower age tertile [34.12 – 52.96 years]			Middle age tertile [53.07 – 61.68 years]			Upper age tertile [61.73 – 85.03 years]		
	Current smokers (n = 46)	Never smokers (n = 46)	P	Current smokers (n = 47)	Never smokers (n = 47)	P	Current smokers (n = 47)	Never smokers (n = 47)	P
Age, mean±SD	47.5 ± 4.6	47.9 ± 4.8	0.12	57.4 ± 2.7	57.5 ± 2.8	0.27	68.6 ± 5.4	68.6 ± 5.3	0.93
(VH-)IVUS segment parameters									
Segment length, mm	45.7 ± 13.1	44.3 ± 12.8	0.64	44.6 ± 17.8	45.2 ± 12.5	0.85	46.0 ± 15.2	44.5 ± 14.5	0.63
Degree of atherosclerosis									
Plaque volume, mm^3	235.5 [127.8 – 322.3]	192.0 [116.9 – 266.8]	0.51	200.9 [140.8 – 309.9]	214.3 [134.1 – 312.5]	0.59	226.0 [147.7 – 306.8]	217.0 [142.8 – 334.2]	0.74
Plaque burden, %	37.8 ± 12.6	33.9 ± 11.1	0.09	38.6 ± 12.7	37.7 ± 10.8	0.67	39.5 ± 12.6	37.4 ± 10.9	0.38
Composition of atherosclerosis									
% FI volume	56.5 ± 10.4	61.4 ± 12.8	0.042	58.6 ± 10.0	59.0 ± 12.4	0.89	57.8 ± 11.1	60.8 ± 12.8	0.22
% FF volume	8.4 [4.8 – 11.6]	9.4 [6.0 – 12.1]	0.69	9.7 [6.1 – 12.7]	8.8 [5.5 – 12.8]	0.14	11.1 [6.3 – 15.3]	8.3 [5.8 – 12.3]	0.08
% NC volume	23.2 ± 9.3	21.0 ± 9.6	0.23	21.6 ± 7.0	21.1 ± 8.8	0.77	20.1 ± 7.4	20.3 ± 8.0	0.98
% DC volume	7.4 [4.9 – 13.1]	7.7 [3.3 – 10.4]	0.28	6.8 [4.5 – 14.1]	9.1 [4.4 – 14.5]	0.59	8.9 [4.5 – 1 4.8]	8.0 [5.0 – 15.0]	0.68
(VH-)IVUS lesion parameters									
≥1 Lesions, n (%)	39 (84.8)	37 (80.4)	0.79	41 (87.2)	43 (91.5)	0.75	40 (85.1)	43 (91.5)	0.51
Presence of high risk lesions, n (%)	33 (71.7)	25 (54.3)	0.15	28 (59.6)	27 (57.4)	1.00	29 (61.7)	31 (66.0)	0.83
High risk lesion type:									
Degree of atherosclerosis									
≥1 Lesion with plaque burden ≥70%, n (%)	10 (21.7)	6 (13.0)	0.34	7 (14.9)	11 (23.4)	0.39	14 (29.8)	10 (21.3)	0.52
≥1 Lesion with MLA ≤4.0mm^2, n (%)	15 (32.6)	12 (26.1)	0.63	11 (23.4)	16 (34.0)	0.38	17 (36.2)	14 (29.8)	0.68
Composition of atherosclerosis									
≥1 TCFA, n (%)	26 (56.5)	20 (43.5)	0.29	20 (42.6)	16 (34.0)	0.54	11 (23.4)	21 (44.7)	0.06

Supplementary Table 2. Odds ratios of current smoking for high plaque burden and for presence of high risk lesion types, stratified on age.

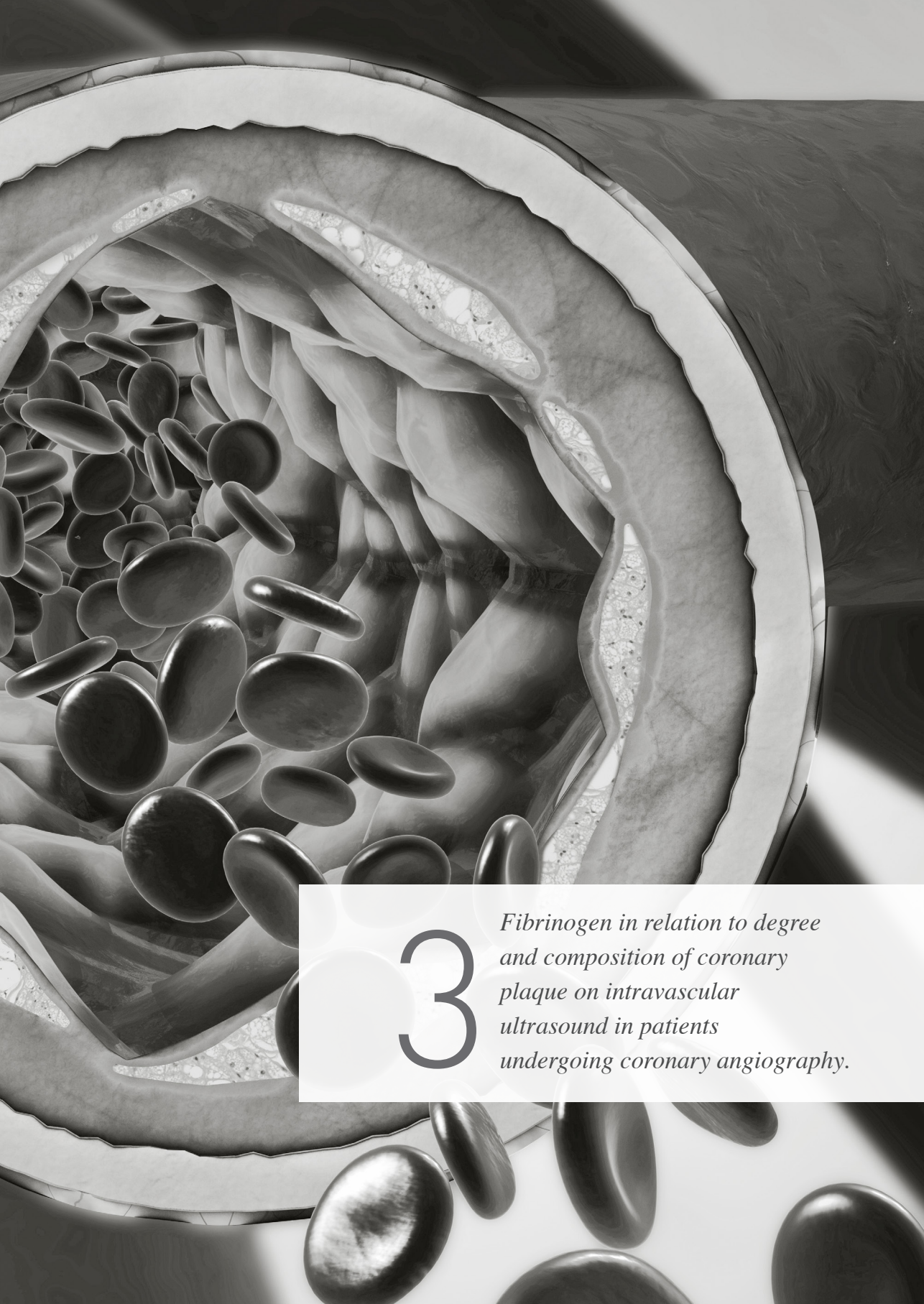
	Lower age tertile [34.12 – 52.96 years]		Middle age tertile [53.07 – 61.68 years]		Upper age tertile [61.73 – 85.03 years]	
	OR (95% CI)	**P**	**OR (95% CI)**	**P**	**OR (95% CI)**	**P**
(VH-)IVUS segment parameters						
Plaque burden						
Below the median	1.00 (reference)		1.00 (reference)		1.00 (reference)	
Above the median	1.67 (0.73 – 3.81)	0.23	1.44 (0.62 – 3.38)	0.40	2.00 (0.86 – 4.67)	0.11
(VH-)IVUS lesion parameters						
≥1 Lesion with plaque burden ≥70%	2.33 (0.60 – 9.02)	0.22	0.50 (0.15 – 1.66)	0.26	1.44 (0.62 – 3.38)	0.40
≥1 Lesion with MLA ≤4.0mm^2	1.43 (0.54 – 3.75)	0.47	0.62 (0.26 – 1.49)	0.28	1.30 (0.57 – 2.97)	0.53
≥1 TCFA	1.75 (0.73 – 4.17)	0.21	1.40 (0.62 – 3.15)	0.42	0.41 (0.17 – 0.99)	0.048

Supplementary Table 3. (VH-)IVUS segment and lesion characteristics in the matched set, stratified on indication.

	ACS patients			SAP patients		
	Current smokers (n = 96)	**Never smokers (n = 96)**	**P**	**Current smokers (n = 44)**	**Never smokers (n = 44)**	**P**
(VH-)IVUS segment parameters						
Segment length, mm	44.9 ± 14.7	43.8 ± 14.0	0.63	46.7 ± 17.0	46.5 ± 11.1	0.97
Degree of atherosclerosis						
Plaque volume, mm^3	215.7 [131.0 – 312.5]	199.7 [113.6 – 282.2]	0.36	226.8 [146.6 – 315.3]	224.9 [148.4 – 327.5]	0.61
Plaque burden, %	38.3 ± 12.8	35.0 ± 11.2	0.049	39.3 ± 12.1	39.3 ± 9.9	0.97
Composition of atherosclerosis						
% FI volume	58.1 ± 9.8	61.1 ± 13.1	0.08	56.8 ± 11.9	58.9 ± 11.4	0.39
% FF volume	9.2 [5.5 – 13.2]	8.6 [5.8 – 12.0]	0.14	10.6 [7.5 – 14.4]	9.3 [5.7 – 12.7]	0.14
% NC volume	22.0 ± 8.1	20.8 ± 9.3	0.34	20.1 ± 7.8	20.7 ± 7.5	0.91
% DC volume	7.8 [4.9 – 13.4]	7.5 [4.3 – 11.8]	0.42	7.6 [3.9 – 16.0]	9.4 [4.5 – 16.5]	0.76
(VH-)IVUS lesion parameters						
≥1 Lesions, n (%)	84 (87.5)	82 (85.4)	0.84	36 (81.8)	41 (93.2)	0.18
Presence of high risk lesions, n (%)	61 (63.5)	60 (62.5)	1.00	29 (65.9)	23 (52.3)	0.26
High risk lesion type:						
Degree of atherosclerosis						
≥1 Lesion with plaque burden ≥70%, n (%)	21 (21.9)	15 (15.6)	0.38	10 (22.7)	12 (27.3)	0.77
≥1 Lesion with MLA ≤4.0mm^2, n (%)	26 (27.1)	29 (30.2)	0.75	17 (38.6)	13 (29.5)	0.52
Composition of atherosclerosis						
≥1 TCFA, n (%)	38 (39.6)	42 (43.8)	0.66	19 (43.2)	15 (34.1)	0.54

Supplementary Table 4. Odds ratios of current smoking for high plaque burden and for presence of high risk lesion types, stratified on indication.

	ACS patients		SAP patients	
	OR (95% CI)	**P**	**OR (95% CI)**	**P**
(VH-)IVUS segment parameters				
Plaque burden				
Below the median	1.00 (reference)		1.00 (reference)	
Above the median	1.88 (1.02 – 3.44)	0.042	1.40 (0.62 – 3.15)	0.42
(VH-)IVUS lesion parameters				
≥1 Lesion with plaque burden ≥70%	1.46 (0.72 – 2.96)	0.29	0.71 (0.23 – 2.25)	0.57
≥1 Lesion with MLA ≤4.0mm^2	0.86 (0.46 – 1.61)	0.63	1.44 (0.62 – 3.38)	0.40
≥1 TCFA	0.84 (0.47 – 1.50)	0.56	1.40 (0.62 – 3.15)	0.42



3

Fibrinogen in relation to degree and composition of coronary plaque on intravascular ultrasound in patients undergoing coronary angiography.

3 Fibrinogen in relation to degree and composition of coronary plaque on intravascular ultrasound in patients undergoing coronary angiography.

Buljubasic N, Akkerhuis KM, Cheng JM, Oemrawsingh RM, Garcia-Garcia HM, de Boer SP, Regar E, van Geuns RM, Serruys PW, Boersma E, Kardys I.

Coron Artery Dis 2017; 28(1): 23-32. doi: 10.1097/MCA.0000000000000442.

Abstract

Rationale: The aim of this study was to provide additional insight into the role of fibrinogen in coronary artery disease by investigating the associations between plasma fibrinogen with both degree and composition of coronary atherosclerosis as determined by virtual histology intravascular ultrasound.

Methods & Results: In 581 patients undergoing coronary angiography for acute coronary syndrome (ACS) or stable angina pectoris, preprocedural blood samples were drawn for fibrinogen, C-reactive protein (CRP), interleukin-6, and plasminogen activator inhibitor-1 measurements, and virtual histology-intravascular ultrasound of a non-culprit coronary artery was performed. The degree [plaque volume, plaque burden (PB), and lesions with PB≥70%] and the composition of coronary atherosclerotic plaque (fibrous, fibrofatty, dense calcium, necrotic core tissue, and thin-cap fibroatheroma lesions) were assessed. Fibrinogen showed a tendency toward a positive association with PB [β (95% CI): 2.55 (-0.52 - 5.61) increase in PB per ln(g/l) fibrinogen, p=0.09], which was driven significantly by an association in the ACS subgroup [β (95% CI): 4.11 (0.01 - 8.21) increase in PB per ln(g/l) fibrinogen, p=0.049]. Fibrinogen was also related to the presence of lesions with PB 70% or more in both the full cohort [OR (95% CI): 2.27 (1.17 - 4.43), p=0.016] and ACS patients [OR

(95% CI): 2.92 (1.17 - 7.29), p=0.022]. All associations were independent of established cardiovascular risk factors, but not CRP. Interleukin-6 and plasminogen activator inhibitor-1 did not provide incremental value to fibrinogen when examining the associations with degree of atherosclerosis. Substantial associations with plaque composition were absent.

Conclusions: Fibrinogen is associated with degree of coronary atherosclerosis, especially in ACS patients. However, whether this association is independent of CRP might be questioned and needs further investigation.

Introduction

Elevated plasma fibrinogen levels have been associated with coronary events both in apparently healthy individuals [1–3] and in patients with manifest coronary artery disease (CAD) [4-7]. Underlying mechanisms that may account for this association have not been fully elucidated as yet, but fibrinogen is known to play an important role in thrombosis [8] and might as well influence the progression of atherosclerotic plaque formation [9,10]. In addition, clinical evidence has shown that fibrinogen is correlated with the severity of atherosclerosis on both coronary angiography and carotid ultrasonography [11-15]. These noninvasive imaging techniques evaluate the unobstructed part of the lumen and have not contributed toward elucidating potential mechanisms of the involvement of fibrinogen in atherosclerosis. In contrast, invasive assessment of coronary plaque by virtual histology-intravascular ultrasound (VH-IVUS) not only accurately quantifies coronary atherosclerosis but also enables in-vivo analysis of coronary plaque composition as well as plaque vulnerability. Thus, this technique provides information on the structure and composition of the arterial wall itself and could add insights into the pathophysiology of coronary atherosclerosis [16].

Until now, the association between fibrinogen and in-vivo coronary plaque characteristics has only been examined in two IVUS studies [17,18]. The first study had a modest study sample size of 60 patients and applied grayscale IVUS to quantify coronary atherosclerosis, which did not allow for assessment of the composition and vulnerability of the coronary plaques [17]. The second study did use VH-IVUS, but again had limited sample size (only 75 patients) [18]. Furthermore, although this study examined necrotic core (NC), it did not assess plaque vulnerability by determining thin-cap fibroatheromas (TCFAs) [18].

Therefore, we investigated the association of plasma fibrinogen with the degree, composition, and vulnerability of coronary atherosclerosis as determined by VH-IVUS, as well as with 1-year cardiovascular outcome, in a relatively large study population consisting of 581 patients undergoing coronary angiography. In addition, to provide a broader view on the pathophysiological relationship between fibrinogen and coronary atherosclerosis, we examined the incremental value of the inflammatory and prothrombotic markers C-reactive protein (CRP), interleukin-6 (IL-6), and plasminogen activator inhibitor-1 (PAI-1) to fibrinogen.

Methods

The rationale and design of the European Collaborative Project on Inflammation and Vascular Wall Remodeling in Atherosclerosis – Intravascular Ultrasound (ATHEROREMO-IVUS) study have been approved by the medical ethics committee of the Erasmus MC (Rotterdam, the Netherlands) and are described in detail elsewhere [19]. The study is registered on ClinicalTrials.gov, number NCT01789411. Briefly, 581 patients undergoing diagnostic coronary angiography or percutaneous coronary intervention for acute coronary syndrome (ACS) or stable angina pectoris (SAP) were included. Written informed consent was obtained from all included patients. Baseline clinical and procedural characteristics were derived from medical records. Blood plasma samples were drawn before the procedure and stored at a temperature of –80°C. Frozen EDTA-plasma samples were transported under controlled conditions to Myriad RBM (Austin, Texas, USA), where fibrinogen, IL-6, and PAI-1 levels were measured successfully in 570 samples (ACS, n=309; SAP, n=261) using a validated, quantitative, multiplexed immunoassay (Custom HumanMAP; Myriad RBM). CRP measurements were performed in serum samples at the clinical laboratory of Erasmus MC using an immunoturbidimetric high-sensitivity assay (Roche Diagnostics Ltd, Rotkreuz, Switzerland) on the Cobas 8000 Modular Analyzer Platform (Roche Diagnostics Ltd). Coefficients of variation were 8% or less, 10% or less, 11% or less, and 4% or less for fibrinogen, IL-6, PAI-1, and CRP, respectively. Following the standard coronary angiography, VH-IVUS data were acquired in a non-culprit coronary artery with the Volcano s5/s5i Imaging System (Volcano Corp., San Diego, California, USA) using a Volcano Eagle Eye Gold IVUS catheter (20 MHz) [19]. The degree and composition of the atherosclerotic plaque

were assessed offline in a dedicated core-lab as described previously [19]. Plaque volume was defined as the total volume of the external elastic membrane occupied by atheroma and was normalized for the length of the imaged segment. Plaque burden (PB) was defined as plaque and media cross-sectional area divided by external elastic membrane cross-sectional area and is presented as a percentage. Atherosclerotic plaque composition was characterized into fibrous (FI), fibrofatty, dense calcium, and NC. Three types of high-risk lesions were identified: (i) VH-IVUS-derived TCFA lesions (presence of >10% confluent NC in direct contact with the lumen); (ii) lesions with large PB (≥70%); and (iii) lesions with a minimal luminal area 4.0mm^2 or less.

Clinical follow-up started at inclusion and lasted 1 year. The primary clinical endpoint consisted of major adverse cardiovascular events (MACEs), which was a composite of death, ACS, or unplanned coronary revascularization [19]. ACS was defined as the clinical diagnosis of non-ST-segment elevation myocardial infarction or unstable angina pectoris [20,21]. Unplanned coronary revascularization was defined as any repeat percutaneous coronary intervention or coronary artery bypass grafting that was not foreseen at the index procedure. Endpoints were adjudicated by a clinical events committee on the basis of original source data.

Normally distributed continuous variables are presented as mean±SD, whereas non-normally distributed continuous variables are presented as median [interquartile range]. Categorical variables are presented as numbers and percentages. Variables with non-normal distributions, determined by visual inspection of the histograms, were natural logarithmically (ln) transformed or were transformed using the square root for further analyses. First, statistical analyses were carried out in the full cohort. Subsequently, they were stratified on indication for catheterization (ACS vs. SAP) to account for potential differences in pathophysiology. Associations of fibrinogen with segment VH-IVUS parameters were examined using linear regression analyses with continuous ln-transformed fibrinogen as the independent variable. The results are presented as β increase in (transformed) segment VH-IVUS parameter per unit increase in ln-transformed fibrinogen concentration, with 95% confidence intervals (CIs). Logistic regression analyses were carried out for associations between fibrinogen and the presence of high-risk lesions with continuous ln-transformed fibrinogen as the independent variable. The results are presented as odds ratios (ORs) per unit increase in ln-transformed fibrinogen concentration, with 95% CIs. All statistical analyses were carried out univariably (model 1) and multivariably (models 2 and 3). Potential confounders were selected on the basis of previous literature (age, sex, smoking, diabetes mellitus, dyslipidemia, hypertension,

indication for catheterization) and entered as covariates into the multivariable models (model 2). Subsequently, CRP was added to the models (model 3) as CRP is the most widely studied inflammatory marker in cardiovascular disease and has been suggested to carry similar predictive value for MACE as fibrinogen [22]. Afterwards, we examined whether the inflammatory markers IL-6 and CRP and the prothrombotic marker PAI-1 provide incremental value to elevated levels of fibrinogen with respect to the degree of atherosclerosis. For this purpose, these markers were dichotomized [CRP and PAI-1: below and above the median; IL-6: detectable (37.7%) vs. nondetectable (62.3%) levels], and degree of atherosclerosis was examined in patients according to both the categories of these markers and dichotomized categories of fibrinogen (above and below median). ANOVA trend tests were used to discern trends across the categories. Finally, we examined associations of fibrinogen with clinical endpoints after 1 year of follow-up with Cox proportional hazard regression analyses.

Results

Baseline clinical and procedural characteristics are presented in Table 1. The mean±SD age of the patients was 61.5±11.4 years (75% were men); 54% of the patients were admitted with ACS and 46% had SAP. The median [interquartile range] fibrinogen level was 3.5 [2.9 - 4.4] g/l in the full cohort. In ACS and SAP patients, this was 3.6 [3.0 - 4.5] and 3.4 [2.8 - 4.3] g/l, respectively (p=0.028).

Table 1. Baseline patient and procedural characteristics.

	Full cohort (n = 570)	ACS patients (n = 309)	SAP patients (n = 261)
Patient characteristics			
Age, years	61.5 ± 11.4	59.7 ± 11.9	63.6 ± 10.3
Male gender, n (%)	430 (75.4)	227 (73.5)	203 (77.8)
Hypertension, n (%)	295 (51.8)	134 (43.4)	161 (61.7)
Dyslipidemia, n (%)	317 (55.6)	137 (44.3)	180 (69.0)
Diabetes mellitus, n (%)	99 (17.4)	40 (12.9)	59 (22.6)
Positive family history, n (%)	293 (51.5)	140 (45.5)	153 (58.6)
Smoking, n (%)	164 (28.8)	115 (37.2)	49 (18.8)
Peripheral artery disease, n (%)	36 (6.3)	12 (3.9)	24 (9.2)
Previous myocardial infarction, n (%)	184 (32.3)	80 (25.9)	104 (39.8)
Previous PCI, n (%)	185 (32.5)	57 (18.4)	128 (49.0)

Table 1 continued.

	Full cohort (n = 570)	ACS patients (n = 309)	SAP patients (n = 261)
Patient characteristics			
Previous CABG, n (%)	18 (3.2)	7 (2.3)	11 (4.2)
Previous stroke, n (%)	23 (4.0)	10 (3.2)	13 (5.0)
History of renal insufficiency, n (%)	32 (5.6)	13 (4.2)	19 (7.3)
Fibrinogen, g/L	3.5 [2.9-4.4]	3.6 [3.0-4.5]	3.4 [2.8-4.3]
Procedural characteristics			
PCI performed, n (%)	501 (87.9)	287 (92.9)	214 (82.0)
Indication for catheterization			
Acute coronary syndrome, n (%)	309 (54.2)	309 (100)	NA
Myocardial infarction, n (%)	159 (27.9)	159 (51.5)	NA
Stable angina pectoris, n (%)	261 (45.8)	NA	261 (100)
Coronary artery disease			
No significant stenosis, n (%)	42 (7.4)	18 (5.8)	24 (9.2)
1-vessel disease, n (%)	301 (52.8)	168 (54.4)	133 (51.0)
2-vessel disease, n (%)	166 (29.1)	88 (28.5)	78 (29.9)
3-vessel disease, n (%)	61 (10.7)	35 (11.3)	26 (10.0)

CABG=coronary artery bypass grafting; NA=not applicable; PCI=percutaneous coronary intervention. Values are mean ± SD, median [interquartile range] or n (%).

The results of the univariable and multivariable analyses for the associations between plasma fibrinogen level and degree and composition of coronary atherosclerosis are shown in Table 2 for the full cohort, in Table 3 for ACS, and in Table 4 for SAP. The degree of atherosclerosis in each group is graphically presented per fibrinogen tertile in Figures 1 and 2. There seemed to be a trend toward higher segment PB (p for trend=0.008 full cohort; p for trend=0.014 ACS subgroup) and a higher prevalence of lesions with PB 70% or more (p for trend=0.002 full cohort; p for trend=0.001 ACS subgroup) with increasing fibrinogen levels. Specifically, fibrinogen showed a tendency toward a positive association with segment PB [β (95% CI): 2.55 (-0.52 - 5.61) increase in %PB per ln(g/l) fibrinogen, p=0.09] in the full cohort (Table 2, model 2, Figure 1), which was driven by a significant association in the ACS subgroup [β (95% CI): 4.11 (0.01 - 8.21) increase in %PB per ln(g/l) fibrinogen, p=0.049] (Table 3, model 2, Figure 1). Fibrinogen was also associated with the presence of lesions with PB 70% or more in both the full cohort [OR (95% CI): 2.27 (1.17 - 4.43), p=0.016] (Table 2, model 2, Figure 2) and ACS patients [OR (95% CI): 2.92 (1.17 - 7.29), p=0.022] (Table 3, model 2, Figure 2). This association did not reach statistical significance in the SAP subgroup [OR (95% CI): 1.69 (0.62 - 4.59), p=0.30] (Table 4, model 2, Figure 2). After additional adjustment for CRP, statistical significance was no longer reached for any of the associations (model 3 in Tables 2-4).

Table 2. Association between fibrinogen and (VH-)IVUS segment and lesion characteristics in the full cohort (n = 570).

(VH-)IVUS segment characteristics	Model 1 (linear regression)[a]		Model 2 (linear regression)[b]		Model 3 (linear regression)[c]	
	β (95% CI)	P-value	β (95% CI)	P-value	β (95% CI)	P-value
Degree of atherosclerosis						
Plaque volume (mm^3) [d]	0.12 (-0.04 - 0.28)	0.15	0.12 (-0.04 - 0.28)	0.15	0.02 (-0.15 - 0.20)	0.79
Plaque burden (%)	2.53 (-0.48 - 5.54)	0.10	2.55 (-0.52 - 5.61)	0.09	0.84 (-2.49 - 4.17)	0.62
Composition of atherosclerosis						
FI volume (mm^3) [d]	1.01 (0.06 - 1.96)	0.037	0.99 (0.01 - 1.96)	0.047	0.46 (-0.60 - 1.52)	0.39
FF volume (mm^3) [d]	0.14 (-0.14 - 0.42)	0.32	0.15 (-0.13 - 0.43)	0.30	0.002 (-0.31 - 0.31)	0.99
NC volume (mm^3) [d]	0.81 (0.11 - 1.51)	0.024	0.83 (0.11 - 1.54)	0.024	0.46 (-0.32 - 1.24)	0.25
DC volume (mm^3) [d]	0.18 (-0.13 - 0.48)	0.25	0.21 (-0.10 - 0.52)	0.18	0.07 (-0.27 - 0.41)	0.68
(VH-)IVUS high-risk lesion characteristics	**Model 1 (logistic regression)[a]**		**Model 2 (logistic regression)[b]**		**Model 3 (logistic regression)[c]**	
	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
Degree of atherosclerosis						
≥ 1 Lesion with MLA ≤ 4.0 mm^2	0.59 (0.32 - 1.07)	0.08	0.59 (0.32 - 1.10)	0.10	0.60 (0.30 - 1.19)	0.14
≥ 1 Lesion with plaque burden ≥70%	2.15 (1.12 - 4.11)	0.021	2.27 (1.17 - 4.43)	0.016	1.90 (0.91 - 3.97)	0.09
Composition of atherosclerosis						
≥ 1 TCFA	1.02 (0.59 - 1.75)	0.95	0.93 (0.53 - 1.63)	0.80	0.83 (0.45 - 1.53)	0.54

CI=confidence interval; DC=dense calcium; FF=fibrofatty; FI=fibrous; MLA=minimal lumen area; NC=necrotic core; OR=odds ratio; TCFA=thin-cap fibroatheroma; VH-IVUS=virtual histology-intravascular ultrasound. β indicates the increase in each (transformed) VH-IVUS segment parameter per unit increase in the logarithm (ln)-transformed fibrinogen level. OR in the presence of high-risk lesions per unit increase in the ln-transformed fibrinogen level. [a]Model 1 (univariable) adjusted for age and sex. [b]Model 2 (multivariable) adjusted for established risk factors (age, sex, smoking, diabetes mellitus, hypertension, and dyslipidemia) and indication for catheterization. [c]Model 3 (multivariable) adjusted for established risk factors, indication for catheterization, and additionally for C-reactive protein. [d]Variables with a non-normal distribution were transformed by the natural ln or square root.

Table 3. Association between fibrinogen and (VH-)IVUS segment and lesion characteristics in acute coronary syndrome patients (n = 309).

(VH-)IVUS segment characteristics	Model 1 (linear regression)[a]		Model 2 (linear regression)[b]		Model 3 (linear regression)[c]	
	β (95% CI)	P-value	β (95% CI)	P-value	β (95% CI)	P-value
Degree of atherosclerosis						
Plaque volume (mm^3) [d]	0.14 (-0.08 - 0.35)	0.21	0.13 (-0.09 - 0.35)	0.25	0.05 (-0.20 - 0.29)	0.71
Plaque burden (%)	4.47 (0.43 - 8.51)	0.030	4.11 (0.01 - 8.21)	0.049	2.26 (-2.28 - 6.81)	0.33
Composition of atherosclerosis						
FI volume (mm^3) [d]	0.99 (-0.28 - 2.26)	0.13	0.90 (-0.39 - 2.19)	0.17	0.32 (-1.11 - 1.75)	0.66
FF volume (mm^3) [d]	0.19 (-0.18 - 0.56)	0.31	0.20 (-0.17 - 0.58)	0.29	0.05 (-0.37 - 0.47)	0.82
NC volume (mm^3) [d]	0.71 (-0.22 - 1.63)	0.13	0.65 (-0.29 - 1.59)	0.17	0.32 (-0.72 - 1.37)	0.54
DC volume (mm^3) [d]	0.25 (-0.15 - 0.65)	0.22	0.26 (-0.15 - 0.67)	0.22	0.16 (-0.29 - 0.62)	0.48
(VH-)IVUS high-risk lesion characteristics	**Model 1 (logistic regression)[a]**		**Model 2 (logistic regression)[b]**		**Model 3 (logistic regression)[c]**	
	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
Degree of atherosclerosis						
≥ 1 Lesion with MLA ≤ 4.0 mm^2	0.75 (0.33 - 1.72)	0.50	0.72 (0.30 - 1.74)	0.47	0.81 (0.30 - 2.14)	0.67
≥ 1 Lesion with plaque burden ≥70%	2.84 (1.17 - 6.91)	0.022	2.92 (1.17 - 7.29)	0.022	2.18 (0.77 - 6.18)	0.14
Composition of atherosclerosis						
≥ 1 TCFA	0.85 (0.42 - 1.75)	0.66	0.77 (0.37 - 1.61)	0.48	0.68 (0.30 - 1.55)	0.36

CI=confidence interval; DC=dense calcium; FF=fibrofatty; FI=fibrous; MLA=minimal lumen area; NC=necrotic core; OR=odds ratio; TCFA=thin-cap fibroatheroma; VH-IVUS=virtual histology-intravascular ultrasound. β indicates the increase in each (transformed) VH-IVUS segment parameter per unit increase in the logarithm (ln)-transformed fibrinogen level. OR in the presence of high-risk lesions per unit increase in the ln-transformed fibrinogen level. [a]Model 1 (univariable) adjusted for age and sex. [b]Model 2 (multivariable) adjusted for established risk factors (age, sex, smoking, diabetes mellitus, hypertension, and dyslipidemia) and indication for catheterization. [c]Model 3 (multivariable) adjusted for established risk factors, indication for catheterization, and additionally for C-reactive protein. [d]Variables with a non-normal distribution were transformed by the natural ln or square root.

Table 4. Association between fibrinogen and (VH-)IVUS segment and lesion characteristics in stable angina pectoris patients (n = 261).

(VH-)IVUS segment characteristics	Model 1 (linear regression)[a]		Model 2 (linear regression)[b]		Model 3 (linear regression)[c]	
	β (95% CI)	P-value	β (95% CI)	P-value	β (95% CI)	P-value
Degree of atherosclerosis						
Plaque volume (mm^3) [d]	0.12 (-0.13 - 0.36)	0.35	0.09 (-0.16 - 0.34)	0.50	-0.005 (-0.27 - 0.26)	0.97
Plaque burden (%)	0.64 (-3.93 - 5.20)	0.78	-0.14 (-4.85 - 4.58)	0.95	-1.32 (-6.29 - 3.65)	0.60
Composition of atherosclerosis						
FI volume (mm^3) [d]	1.17 (-0.31 - 2.65)	0.12	1.02 (-0.50 - 2.54)	0.19	0.62 (-0.99 - 2.22)	0.45
FF volume (mm^3) [d]	0.14 (-0.28 - 0.57)	0.51	0.05 (-0.38 - 0.49)	0.81	-0.06 (-0.52 - 0.40)	0.80
NC volume (mm^3) [d]	1.02 (-0.08 - 2.12)	0.07	0.91 (-0.22 - 2.04)	0.12	0.59 (-0.61 - 1.79)	0.33
DC volume (mm^3) [d]	0.16 (-0.32 - 0.63)	0.51	0.10 (-0.39 - 0.59)	0.68	-0.04 (-0.55 - 0.47)	0.88
(VH-)IVUS high-risk lesion characteristics	**Model 1 (logistic regression)[a]**		**Model 2 (logistic regression)[b]**		**Model 3 (logistic regression)[c]**	
	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
Degree of atherosclerosis						
≥ 1 Lesion with MLA ≤ 4.0 mm^2	0.47 (0.19 - 1.15)	0.10	0.41 (0.16 - 1.05)	0.06	0.41 (0.15 - 1.09)	0.07
≥ 1 Lesion with plaque burden ≥70%	1.81 (0.69 - 4.77)	0.23	1.69 (0.62 - 4.59)	0.30	1.61 (0.56 - 4.66)	0.38
Composition of atherosclerosis						
≥ 1 TCFA	1.13 (0.48 - 2.65)	0.79	1.07 (0.44 - 2.59)	0.89	0.98 (0.38 - 2.52)	0.97

CI=confidence interval; DC=dense calcium; FF=fibrofatty; FI=fibrous; MLA=minimal lumen area; NC=necrotic core; OR=odds ratio; TCFA=thin-cap fibroatheroma; VH-IVUS=virtual histology-intravascular ultrasound. β indicates the increase in each (transformed) VH-IVUS segment parameter per unit increase in the logarithm (ln)-transformed fibrinogen level. OR in the presence of high-risk lesions per unit increase in the ln-transformed fibrinogen level. [a]Model 1 (univariable) adjusted for age and sex. [b]Model 2 (multivariable) adjusted for established risk factors (age, sex, smoking, diabetes mellitus, hypertension, and dyslipidemia) and indication for catheterization. [c]Model 3 (multivariable) adjusted for established risk factors, indication for catheterization, and additionally for C-reactive protein. [d]Variables with a non-normal distribution were transformed by the natural ln or square root.

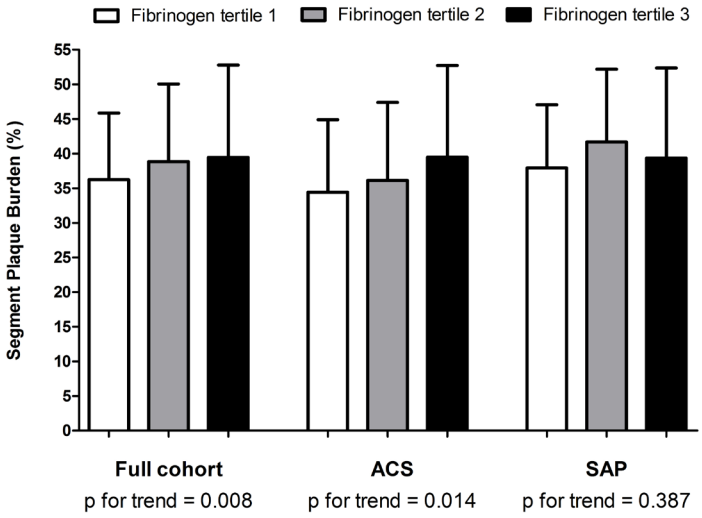


Figure 1. Segment plaque burden (%) in relation to fibrinogen tertiles.

ACS=acute coronary syndrome; SAP=stable angina pectoris. Observed absolute values of segment plaque burden (%) per fibrinogen tertile in the full cohort and stratified groups. The ANOVA trend test was used to calculate the p-values for trend and thus to discern trends across the categories.

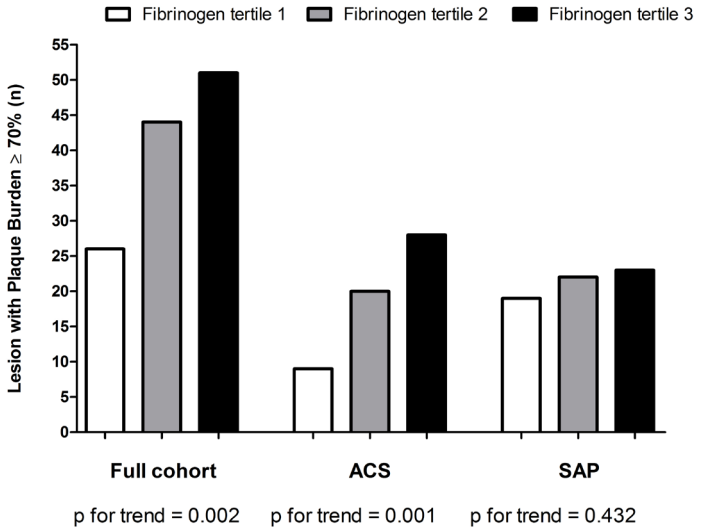


Figure 2. Presence of lesions with plaque burden 70% or more in relation to fibrinogen tertiles.

ACS=acute coronary syndrome; SAP=stable angina pectoris. Observed numbers of lesions with plaque burden 70% or more per fibrinogen tertile in the full cohort and stratified groups. The ANOVA trend test was used to calculate the p-values for trend and thus to discern trends across the categories.

With respect to the composition and vulnerability of atherosclerosis, in the full cohort, fibrinogen was associated positively with NC volume [β (95% CI): 0.83 (0.11 - 1.54)mm^3 increase in NC volume per ln(g/l) fibrinogen, p=0.024] and FI tissue [β (95% CI): 0.99 (0.01 - 1.96)mm^3 increase in FI volume per ln(g/l) fibrinogen, p=0.047] (Table 2, model 2). However, fibrinogen was not associated with the presence of VH-TCFA lesions (i.e. rupture-prone coronary plaques). No associations with composition were present in the ACS and SAP subgroups. After additional adjustment for CRP, again, statistical significance was no longer reached for any of the associations (model 3 in Tables 2-4).

We have described the individual associations of CRP, IL-6, and PAI-1 with the degree, composition, and vulnerability of atherosclerosis in this cohort in earlier reports [23–25]. Briefly, we found that CRP was associated with degree, but not composition or vulnerability of atherosclerosis, and IL-6 and PAI-1 did not show significant associations with any of the plaque characteristics. In the present study, trend analysis showed that patients with both elevated fibrinogen and CRP levels have higher segment PB (p for trend=0.041) and a higher prevalence of lesions with PB 70% or more (p for trend=0.090), especially in the ACS subgroup (p for trend=0.001) (Figures 3 and 4). Such a trend could not be found for fibrinogen in combination with IL-6 or PAI-1 (Figures 5-8).

With respect to clinical outcome (results not shown), in the full cohort as well as in the individual ACS and SAP subgroups, no association was found between fibrinogen and MACE. A borderline significant univariable association between fibrinogen and the secondary, composite endpoint of death, or ACS was present in the full cohort [HR (95% CI): 2.69 (0.99 - 7.30), p=0.052]. After adjustment for age and sex, the association became nonsignificant, although the effect estimate remained materially the same [HR (95% CI): 2.36 (0.80 - 6.93), p=0.12]. With further adjustment for CRP, the HR became closer to the null [HR (95% CI): 0.73 (0.20 - 2.64), p=0.64].

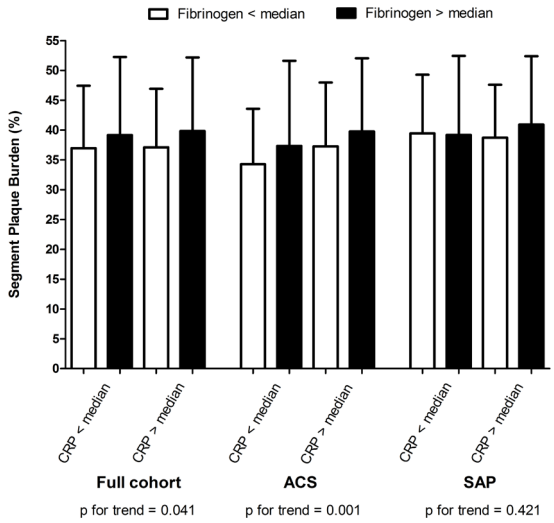


Figure 3. Segment plaque burden (%) in relation to combined dichotomized CRP and fibrinogen levels.

ACS=acute coronary syndrome; CRP=C-reactive protein; SAP=stable angina pectoris. Observed absolute values of segment plaque burden (%) per category of CRP (above or under median) and fibrinogen (above or under median) together in the full cohort and stratified groups. The ANOVA trend test was used to calculate the p-values for trend and thus to discern trends across the categories.

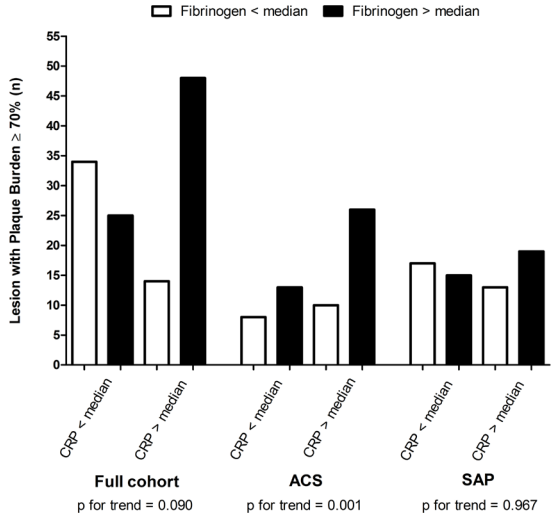


Figure 4. Presence of lesions with plaque burden 70% or more in relation to combined dichotomized CRP and fibrinogen levels.

ACS=acute coronary syndrome; CRP=C-reactive protein; SAP=stable angina pectoris. Observed numbers of lesions with plaque burden 70% or more per category of CRP (above or under median) and fibrinogen (above or under median) together in the full cohort and stratified groups. The ANOVA trend test was used to calculate the p-values for trend and thus to discern trends across the categories.

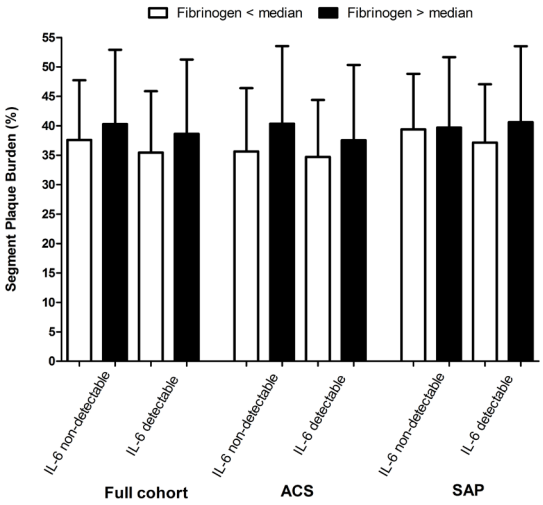


Figure 5. Segment plaque burden (%) in relation to combined dichotomized IL-6 and fibrinogen levels.

ACS=acute coronary syndrome; IL-6=interleukin-6; SAP=stable angina pectoris. Observed absolute values of segment plaque burden (%) per category of IL-6 (detectable or nondetectable) and fibrinogen (above or under median) together in the full cohort and stratified groups.

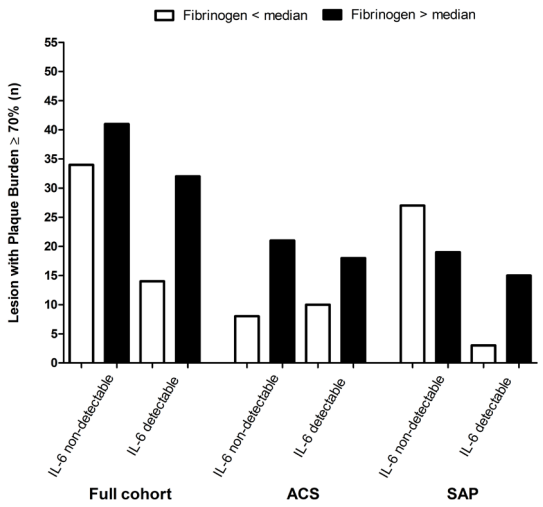


Figure 6. Presence of lesions with plaque burden 70% or more in relation to combined dichotomized IL-6 and fibrinogen levels.

ACS=acute coronary syndrome; IL-6=interleukin-6; SAP=stable angina pectoris. Observed numbers of lesions with plaque burden 70% or more per category of IL-6 (detectable or nondetectable) and fibrinogen (above or under median) together in the full cohort and stratified groups.

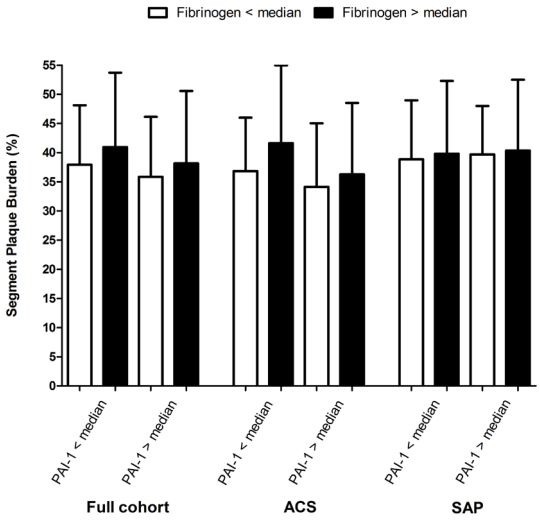


Figure 7. Segment plaque burden (%) in relation to combined dichotomized PAI-1 and fibrinogen levels.

ACS=acute coronary syndrome; PAI-1=plasminogen activator inhibitor type-1; SAP=stable angina pectoris. Observed absolute values of segment plaque burden (%) per category of PAI-1 (above or under median) and fibrinogen (above or under median) together in the full cohort and stratified groups.

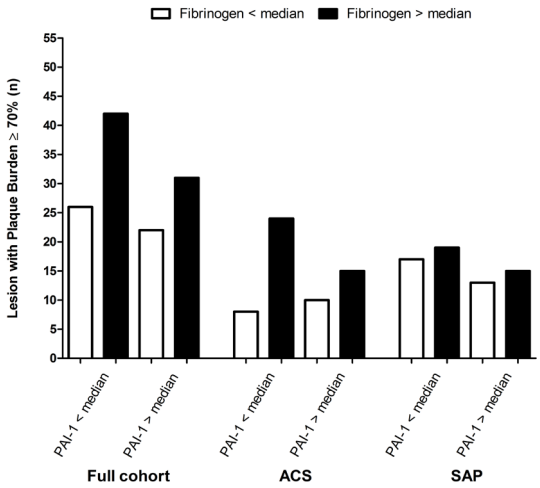


Figure 8. Presence of lesions with plaque burden 70% or more in relation to combined dichotomized PAI-1 and fibrinogen levels.

ACS=acute coronary syndrome; PAI-1=plasminogen activator inhibitor type-1; SAP=stable angina pectoris. Observed numbers of lesions with plaque burden 70% or more per category of PAI-1 (above or under median) and fibrinogen (above or under median) together in the full cohort and stratified groups.

Discussion

This is the first large study that has investigated the association between plasma fibrinogen level and the degree, composition, and vulnerability of coronary atherosclerotic plaque as assessed by VH-IVUS. Thus, our study provides additional insight into the nature of the relationship between fibrinogen and CAD. We found that fibrinogen was associated with PB and presence of large lesions, both significantly driven by patients presenting with ACS. Conversely, we could not find substantial associations between fibrinogen and atherosclerotic plaque composition, including VH-IVUS-derived TCFA lesions (i.e. rupture-prone coronary plaques). The latter agrees with an earlier study, which could not find an association between fibrinogen and rupture-prone carotid plaques on ultrasonography [26]. Altogether, these findings might indicate that fibrinogen plays a pathogenic role in the progression of atherosclerotic plaque formation rather than in plaque vulnerability, especially in ACS patients.

Although several studies using different modalities have examined the association between fibrinogen and atherosclerosis, so far, only two studies have applied the IVUS technique [17,18]. Hartmann et al. [17] examined this association with grayscale IVUS in only 60 patients and concluded that fibrinogen levels correlate with plaque progression on IVUS, which is in line with our findings. Yet, the grayscale IVUS technique did not enable examination of plaque composition or plaque vulnerability. The other study published recently by Corban et al. [18], used the VH-IVUS technique as we did, but the study size was again modest with 75 patients. They concluded that fibrinogen degradation products are associated with larger plaques that have a larger NC. Although they did not stratify their study population by clinical diagnosis (ACS vs. SAP), their findings concur with our results in the full cohort. Here, the authors suggest that fibrinogen may play a role in plaque vulnerability, which should be confirmed in studies that determined TCFAs. However, as the study population was relatively small and consisted of patients with nonobstructive CAD, this study contained a low number of TCFAs. Therefore, the authors could not investigate further whether fibrinogen is associated with plaque vulnerability. In our study, with a relatively large sample size, we could examine the relationship between fibrinogen and TCFAs, but the results did not confirm a relationship with fibrinogen. Therefore, we hypothesize that fibrinogen might rather play a pathogenic role in atherosclerotic plaque formation than in plaque vulnerability.

A potential association of fibrinogen with degree of coronary plaque is in line with earlier studies, proposing a mechanism by which fibrinogen, as a major determinant of thrombus

formation, could directly contribute toward the progression of coronary atherosclerosis [8,27]. Fibrinogen is believed to be involved in an underlying process of multiple consecutive mild episodes of mural thrombosis in response to subclinical rupture [28], leading to repeated incorporation of small thrombi and eventually resulting in gradual progression of an atherosclerotic plaque [29]. This hypothesis is further supported by clinical evidence showing that high fibrinogen levels are positively correlated with the extent of atheroma on coronary angiography [11,14,30,31]. Another study that measured the progress of carotid atherosclerosis by high-resolution duplex ultrasound found a temporal relationship between fibrinogen and advanced atherosclerosis rather than with early (inflammatory) stages of the disease [13]. In addition, immunohistochemical studies have shown the presence of fibrinogen in atherosclerotic plaques, indicating that fibrinogen may be directly involved in plaque progression [32].

According to autopsy studies [33], repeated, healed silent plaque ruptures with incorporated organized thrombi are predominantly found in patients with ACS, indicating episodic plaque growth and thereby contributing toward the progression of advanced atherosclerotic plaques in these patients. This might explain the fact that we found a positive association of fibrinogen with degree of coronary atherosclerosis in ACS patients in particular. Rupture-prone, soft, lipid-rich plaques have previously been found to be more common in patients presenting with ACS compared with SAP patients [34]. Taken together with reported findings of increased fibrinogen levels in patients with established advanced CAD [35,36], the unstable lipid-rich plaques in ACS patients may be more thrombogenic than stable FI plaques in SAP patients [37,38]. Thus, ACS patients with increased fibrinogen levels are likely to have experienced multiple consecutive mild episodes of mural thrombosis previously, eventually leading to a higher degree of atherosclerotic plaque compared with SAP patients.

Another prothrombotic factor that we investigated was PAI-1, which has led to paradoxical results in previous studies [39]. Conflicting results exist with respect to the role of PAI-1 in both promoting and preventing plaque development because of its complex multilevel functions [39,40]. We could not find higher or lower degree of coronary plaque in patients with elevated levels of both fibrinogen and PAI-1.

The observed associations of fibrinogen with degree of atherosclerosis were not independent of CRP. CRP has been associated with the presence and degree of atherosclerosis [23,41], and both fibrinogen and CRP are linked to vascular inflammation [42,43]. In line with our current results, Hartmann et al. [17] found a positive, but not independent, correlation between fibrinogen levels and plaque progression on IVUS. Furthermore, in our study, a higher degree

of atherosclerosis was present in patients with both high levels of CRP and high levels of fibrinogen, suggesting a synergistic effect on coronary atherosclerosis.

Inflammatory processes result in the release of numerous mediators, including IL-6, which is the principal procoagulant cytokine [42]. Although the exact interaction is unknown, it is suggested that IL-6 on its part can increase plasma concentrations of both fibrinogen and CRP, which further amplify inflammatory and procoagulant responses [42]. However, as we have reported earlier, in the current cohort, we found no associations between IL-6 and coronary plaque characteristics [25]. Also, in the current investigation, we could not find any trend toward a higher degree of coronary atherosclerosis in patients with both high fibrinogen and high IL-6 levels.

Finally, our data suggest that fibrinogen may be associated with the incidence of all-cause mortality or ACS. The fact that the association became nonsignificant after adjustment for age and sex may have resulted from limited statistical power (56 events after 1-year of follow-up) as the effect estimate remained large after adjustment. However, after additional adjustment for CRP, the associations disappeared completely. Again, common inflammatory grounds may have played a role here. Although population-based studies have shown that fibrinogen is associated with adverse outcome independent of CRP [1], studies in patients with known CAD are fewer in number [4,5,44] and not all of them have taken CRP into account.

Some limitations of this study must be acknowledged. First, the possibility that fibrinogen as an acute-phase reactant may be the result of clinical presentation rather than its cause, particularly in patients with ACS, cannot be excluded. Our study design does not enable causal inference. Nevertheless, our study does provide novel data on fibrinogen in relation to in-vivo assessment of the arterial wall (i.e. more accurate measures of coronary atherosclerosis) [45] and may thus serve to be hypothesis generating. Second, VH-IVUS imaging data were acquired in a non-culprit coronary artery segment only. This approach was based on the hypothesis that such a non-culprit target segment adequately reflects the patient's overall state of coronary wall pathophysiology of the larger coronary vasculature [19]. This hypothesis has been confirmed by several IVUS studies that showed that the coronary wall of a non-culprit segment in a single vessel does reflect larger coronary disease burden and is associated with subsequent cardiovascular events [46,47].

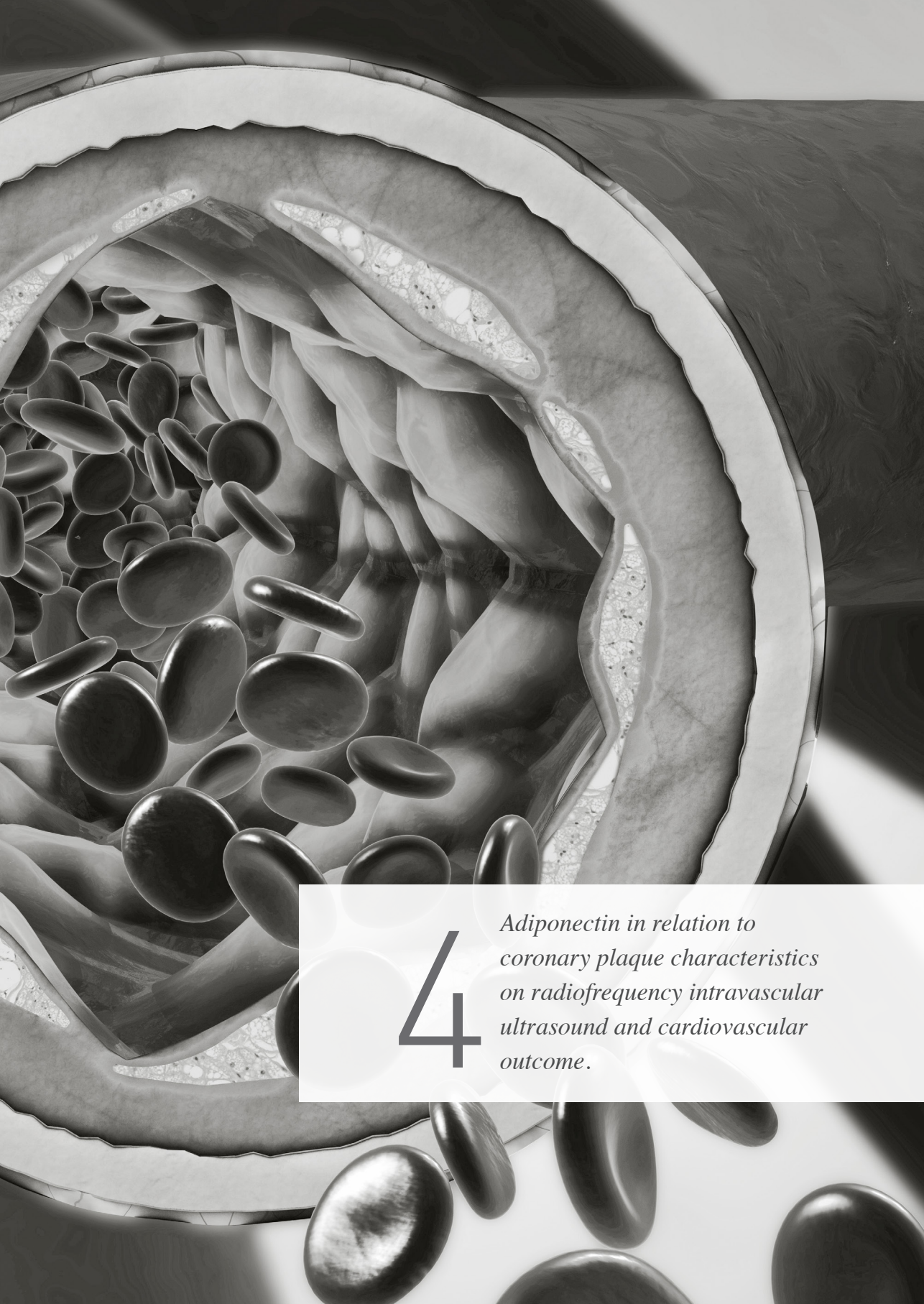
In conclusion, our findings support the hypothesis that fibrinogen is associated with coronary atherosclerotic PB, especially in patients presenting with ACS. However, whether this association is independent of CRP may be questioned and warrants further investigation.

References

1. Danesh J. et al. Plasma fibrinogen level and the risk of major cardiovascular diseases and nonvascular mortality: an individual participant meta-analysis. JAMA 2005; 294: 1799–1809.
2. Kannel W.B. et al. Fibrinogen and risk of cardiovascular disease. The Framingham Study. JAMA 1987; 258: 1183–1186.
3. Park C.S. et al. Relation between C-reactive protein, homocysteine levels, fibrinogen, and lipoprotein levels and leukocyte and platelet counts, and 10-year risk for cardiovascular disease among healthy adults in the USA. Am J Cardiol 2010; 105: 1284–1288.
4. Becker R.C. et al. Prognostic value of plasma fibrinogen concentration in patients with unstable angina and non-Q-wave myocardial infarction (TIMI IIIB Trial). Am J Cardiol 1996; 78: 142–147.
5. Toss H. et al. Prognostic influence of increased fibrinogen and C-reactive protein levels in unstable coronary artery disease. Circulation 1997; 96: 4204–4210.
6. Sinning J.M. et al. Impact of C-reactive protein and fibrinogen on cardiovascular prognosis in patients with stable angina pectoris: the AtheroGene study. Eur Heart J 2006; 27: 2962–2968.
7. Ndrepepa G. et al. Relation of fibrinogen level with cardiovascular events in patients with coronary artery disease. Am J Cardiol 2013; 111: 804–810.
8. De Moerloose P. et al. Fibrinogen and the risk of thrombosis. Semin Thromb Hemost 2010; 36: 7–17.
9. Yee K.O. and Schwartz S.M. Why atherosclerotic vessels narrow: the fibrin hypothesis. Thromb Haemost 1999; 82: 762–771.
10. Koenig W. Fibrin(ogen) in cardiovascular disease: an update. Thromb Haemost 2003; 89: 601–609.
11. Handa K. et al. Plasma fibrinogen levels as an independent indicator of severity of coronary atherosclerosis. Atherosclerosis 1989; 77: 209–213.
12. Levenson J. et al. Fibrinogen and silent atherosclerosis in subjects with cardiovascular risk factors. Arterioscler Thromb Vasc Biol 1995; 15: 1263–1268.
13. Willeit J. et al. Distinct risk profiles of early and advanced atherosclerosis: prospective results from the Bruneck study. Arterioscler Thromb Vasc Biol 2000; 20: 529–537.
14. Zhang Y. et al. Fibrinogen and the severity of coronary atherosclerosis among adults with and without statin treatment: lipid as a mediator. Heart Lung Circ 2016; 25: 558–567.
15. Zhang Y. et al. Higher fibrinogen level is independently linked with the presence and severity of new-onset coronary atherosclerosis among Han Chinese population. PLoS One 2014; 9: e113460.
16. Garcia-Garcia H.M. et al. Imaging of coronary atherosclerosis: intravascular ultrasound. Eur Heart J 2010; 31: 2456–2469.
17. Hartmann M. et al. Relation between lipoprotein(a) and fibrinogen and serial intravascular ultrasound plaque progression in left main coronary arteries. J Am Coll Cardiol 2006; 48: 446–452.
18. Corban M.T. et al. Elevated levels of serum fibrin and fibrinogen degradation products are independent predictors of larger coronary plaques and greater plaque necrotic core. Circ J 2016; 80: 931–937.
19. De Boer S.P. et al. Relation of genetic profile and novel circulating biomarkers with coronary plaque phenotype as determined by intravascular ultrasound: rationale and design of the ATHEROREMO-IVUS study. EuroIntervention 2014; 10: 953–960.
20. Van de Werf F. et al. Management of acute myocardial infarction in patients presenting with persistent ST-segment elevation: the Task Force on the Management of ST-segment Elevation Acute Myocardial Infarction of the European Society of Cardiology. Eur Heart J 2008; 29: 2909–2945.

21. Hamm C.W. et al. ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: the Task Force for the management of acute coronary syndromes (ACS) in patients presenting without persistent ST-segment elevation of the European Society of Cardiology (ESC). Eur Heart J 2011; 32: 2999–3054.
22. Kaptoge S. et al. C-reactive protein, fibrinogen, and cardiovascular disease prediction. N Engl J Med 2012; 367: 1310–1320.
23. Cheng J.M. et al. Relation of C-reactive protein to coronary plaque characteristics on grayscale, radiofrequency intravascular ultrasound, and cardiovascular outcome in patients with acute coronary syndrome or stable angina pectoris (from the ATHEROREMO-IVUS study). Am J Cardiol 2014; 114: 1497–1503.
24. Battes L.C. et al. Circulating acute phase proteins in relation to extent and composition of coronary atherosclerosis and cardiovascular outcome: results from the ATHEROREMO-IVUS study. Int J Cardiol 2014; 177: 847–853.
25. Battes L.C. et al. Circulating cytokines in relation to the extent and composition of coronary atherosclerosis: results from the ATHEROREMO-IVUS study. Atherosclerosis 2014; 236: 18–24.
26. Kofoed S.C. et al. Fibrinogen predicts ischaemic stroke and advanced atherosclerosis but not echolucent, ruptureprone carotid plaques: the Copenhagen City Heart Study. Eur Heart J 2003; 24: 567–576.
27. Bini A. and Kudryk B.J. Fibrinogen in human atherosclerosis. Ann N Y Acad Sci 1995; 748: 461–471; discussion 471–463.
28. Zaman A.G. et al. The role of plaque rupture and thrombosis in coronary artery disease. Atherosclerosis 2000; 149: 251–266.
29. Stary H.C. et al. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation 1995; 92: 1355–1374.
30. Broadhurst P. et al. Fibrinogen, factor VII clotting activity and coronary artery disease severity. Atherosclerosis 1990; 85: 169–173.
31. Tataru M.C. et al. Plasma fibrinogen in relation to the severity of arteriosclerosis in patients with stable angina pectoris after myocardial infarction. Coron Artery Dis 2001; 12: 157–165.
32. Bini A. et al. Identification and distribution of fibrinogen, fibrin, and fibrin(ogen) degradation products in atherosclerosis. Use of monoclonal antibodies. Arteriosclerosis 1989; 9: 109–121.
33. Burke AP, Kolodgie FD, Farb A, Weber DK, Malcom GT, Smialek J, et al. Healed plaque ruptures and sudden coronary death: evidence that subclinical rupture has a role in plaque progression. Circulation 2001; 103: 934–940.
34. Thieme T. et al. Angioscopic evaluation of atherosclerotic plaques: validation by histomorphologic analysis and association with stable and unstable coronary syndromes. J Am Coll Cardiol 1996; 28: 1–6.
35. Kruskal J.B. et al. Fibrin and fibrinogenrelated antigens in patients with stable and unstable coronary artery disease. N Engl J Med 1987; 317: 1361–1365.
36. ECAT angina pectoris study: baseline associations of haemostatic factors with extent of coronary arteriosclerosis and other coronary risk factors in 3000 patients with angina pectoris undergoing coronary angiography. Eur Heart J 1993; 14: 8–17.
37. Fernandez-Ortiz A. et al. Characterization of the relative thrombogenicity of atherosclerotic plaque components: implications for consequences of plaque rupture. J Am Coll Cardiol 1994; 23: 1562–1569.
38. Ando H. et al. Comparison of tissue characteristics between acute coronary syndrome and stable angina pectoris. An integrated backscatter intravascular ultrasound analysis of culprit and non-culprit lesions. Circ J 2011; 75: 383–390.

39. Diebold I. et al. The 'PAI-1 paradox' in vascular remodeling. Thromb Haemost 2008; 100: 984–991.
40. Ploplis V.A. Effects of altered plasminogen activator inhibitor-1 expression on cardiovascular disease. Curr Drug Targets 2011; 12: 1782–1789.
41. Elias-Smale S.E. et al. C-reactive protein is related to extent and progression of coronary and extra-coronary atherosclerosis; results from the Rotterdam study. Atherosclerosis 2007; 195: E195–E202.
42. Willerson J.T. and Ridker P.M. Inflammation as a cardiovascular risk factor. Circulation 2004; 109: 2–10.
43. Kubo T. et al. High-sensitivity C-reactive protein and plaque composition in patients with stable angina pectoris: a virtual histology intravascular ultrasound study. Coron Artery Dis 2009; 20: 531–535.
44. Acevedo M. et al. Fibrinogen: associations with cardiovascular events in an outpatient clinic. Am Heart J 2002; 143: 277–282.
45. Nasu K. et al. Accuracy of in vivo coronary plaque morphology assessment: a validation study of in vivo virtual histology compared with in vitro histopathology. J Am Coll Cardiol 2006; 47: 2405–2412.
46. Cheng J.M. et al. In vivo detection of high-risk coronary plaques by radiofrequency intravascular ultrasound and cardiovascular outcome: results of the ATHEROREMO-IVUS study. Eur Heart J 2014; 35: 639–647.
47. Nicholls S.J. et al. Intravascular ultrasound-derived measures of coronary atherosclerotic plaque burden and clinical outcome. J Am Coll Cardiol 2010; 55: 2399–2407.



4

Adiponectin in relation to coronary plaque characteristics on radiofrequency intravascular ultrasound and cardiovascular outcome.

Adiponectin in relation to coronary plaque characteristics on radiofrequency intravascular ultrasound and cardiovascular outcome.

Marino BCA, Buljubasic N, Akkerhuis M, Cheng JM, Garcia-Garcia HM, Regar E, Geuns RV, Serruys PW, Boersma E, Kardys I.

Arq Bras Cardiol 2018; 111(3): 345-353. doi: 10.5935/abc.20180172.

Abstract

Rationale: Prospective data on the associations of adiponectin with in-vivo measurements of degree, phenotype and vulnerability of coronary atherosclerosis are currently lacking. The objective is to investigate the association of plasma adiponectin with virtual histology intravascular ultrasound (VH-IVUS)-derived measures of atherosclerosis and with major adverse cardiac events (MACE) in patients with established coronary artery disease.

Methods & Results: In 2008-2011, VH-IVUS of a non-culprit non-stenotic coronary segment was performed in 581 patients undergoing coronary angiography for acute coronary syndrome (ACS, n=318) or stable angina pectoris (SAP, n=263) from the atherosclerosis-intravascular ultrasound (ATHEROREMO-IVUS) study. Blood was sampled prior to coronary angiography. Coronary plaque burden, tissue composition, high-risk lesions, including VH-IVUS-derived thin-cap fibroatheroma (TCFA), were assessed. All-cause mortality, ACS, unplanned coronary revascularization were registered during a 1-year-follow-up. All statistical tests were two-tailed and p-values <0.05 were considered statistically significant. In the full cohort, adiponectin levels were not associated with plaque burden, nor with the

various VH-tissue types. In SAP patients, adiponectin levels (median [IQR]: 2.9 [1.9 - 3.9] μg/mL) were positively associated with VH-IVUS derived TCFA lesions, (OR (95% CI): 1.78 (1.06 - 3.00), p=0.030), and inversely associated with lesions with minimal luminal area (MLA) ≤4.0 mm2 (OR (95% CI): 0.55 (0.32 - 0.92), p=0.025). In ACS patients, adiponectin levels (median [IQR]: 2.9 [1.8 - 4.1] μg/mL) were not associated with plaque burden, nor with tissue components. Positive association of adiponectin with death was present in the full cohort (HR (95% CI): 2.52 (1.02 - 6.23), p=0.045) and (borderline) in SAP patients (HR (95% CI): 8.48 (0.92 - 78.0), p=0.058). In ACS patients, this association lost statistical significance after multivariable adjustment (HR (95% CI): 1.87 (0.67 - 5.19), p=0.23).

Conclusions: In the full cohort, adiponectin levels were associated with death but not with VH-IVUS atherosclerosis measures. In SAP patients, adiponectin levels were associated with VH-IVUS-derived TCFA lesions. Altogether, substantial role for adiponectin in plaque vulnerability remains unconfirmed.

Introduction

Coronary plaque rupture has been described as the main mechanism through which mildly stenotic coronary atherosclerosis can lead to acute coronary thrombosis and myocardial infarction [1]. High-risk plaques that are vulnerable to such rupture demonstrate distinct morphological characteristics [2]. They can be differentiated from lesions responsible for stable coronary artery disease (CAD) by their large necrotic cores, thin inflamed fibrous caps, and positive remodeling [2]. Because plaque vulnerability is associated with inflammation, neovascularization, and necrotic core formation, circulating mediators of these processes may aid in detection of high-risk patients and therefore warrant investigation [3]. One important inflammatory mediator of CAD is adiponectin. Adiponectin is a protein mainly produced in white adipose tissue, involved in several antioxidant, anti-inflammatory, and anti-atherosclerotic processes [4-6]. Several studies have demonstrated associations of adiponectin with clinical adverse coronary events [7-10]. Yet, prospective data on the associations of adiponectin with *in-vivo* measurements of degree, phenotype and vulnerability of coronary atherosclerosis are currently lacking. To further elucidate the pathophysiology of adiponectin in patients with established CAD, we investigated the association of adiponectin with virtual

histology intravascular ultrasound (VH-IVUS)-derived measures of degree and composition of coronary atherosclerosis, and with major adverse cardiac events (MACE), in patients undergoing coronary angiography.

Methods

The design of The European Collaborative Project on Inflammation and Vascular Wall Remodeling in Atherosclerosis – Intravascular Ultrasound (ATHEROREMO-IVUS) study has been described in detail elsewhere [11,12]. In brief, 581 patients who underwent diagnostic coronary angiography or percutaneous coronary intervention (PCI) for acute coronary syndrome (ACS, n=318) or stable angina pectoris (SAP, n=263) have been included between 2008 and 2011 in the Erasmus University Medical Center (Erasmus MC), Rotterdam, the Netherlands. The ATHEROREMO-IVUS study was approved by the medical ethics committee of Erasmus MC. The study was performed in accordance with the criteria described in the declaration of Helsinki. Written informed consent was obtained from all included patients. This study is registered in ClinicalTrials.gov, number NCT01789411.

Blood samples for biomarker measurements were drawn from the arterial sheath prior to coronary angiography and were available in 570 patients for the current study. The blood samples were stored at the clinical laboratory of Erasmus MC at a temperature of -80°C within 2 hours after blood collection. C-reactive protein (CRP) was measured in serum samples using an immunoturbidimetric high sensitivity assay (Roche Diagnostics Ltd., Rotkreuz, Switzerland) on the Roche Cobas 8000 modular analyzer platform. These analyses were performed in the clinical laboratory of Erasmus MC. Frozen EDTA plasma samples were transported under controlled conditions (at a temperature of -80°C) to Myriad RBM, Austin, Texas, USA, where adiponectin was measured using a validated multiplex assay (Custom Human Map, Myriad RBM).

Following the standard coronary angiography or PCI procedure, intravascular ultrasound (IVUS) imaging took place in a target segment of a non-culprit coronary artery which was required to be at least 40mm in length and without significant luminal narrowing (<50% stenosis) as assessed by on-line angiography. Selection of the non-culprit vessel was predefined in the study protocol. The order of preference for selection of the non-culprit vessel was: (1) left anterior descending (LAD) artery; (2) right coronary artery (RCA); (3) left circumflex

(LCX) artery. All IVUS data were acquired with the Volcano s5/s5i Imaging System (Volcano Corp., San Diego, California) using a Volcano Eagle Eye Gold IVUS catheter (20MHz). An automatic pullback system was used with a standard pullback speed of 0.5mm per second. The IVUS images were analyzed offline by an independent core laboratory (Cardialysis BV, Rotterdam, the Netherlands) blinded for clinical and biomarker data. The IVUS gray-scale and IVUS radiofrequency analyses, also known as VH-IVUS, were performed using pcVH 2.1 and qVH (Volcano Corp., San Diego, California) software. The external elastic membrane and luminal borders were contoured for each frame (median inter-slice distance, 0.40 mm). Extent and phenotype of the atherosclerotic plaque were assessed. Plaque volume was defined as the total volume of the external elastic membrane occupied by atheroma [13]. Plaque burden was defined as plaque and media cross-sectional area divided by external elastic membrane cross-sectional area and is presented as a percentage. The composition of the atherosclerotic plaque was characterized into four different tissue types: fibrous, fibrofatty, dense calcium and necrotic core [14]. A coronary lesion was defined as a segment with a plaque burden of more than 40% in at least three consecutive frames.

The following types of VH-IVUS high-risk lesions were identified:

(1) Thin-cap fibroatheroma (TCFA) lesions: lesions with presence of >10% confluent necrotic core in direct contact with the lumen [15,16]
(2) TCFA lesions with a plaque burden of at least 70%
(3) Lesions with a plaque burden of at least 70%
(4) Lesions with a minimal luminal area (MLA) of ≤4.0 mm^2 [11]

Follow-up started at inclusion and lasted 1 year. Post-discharge survival status was obtained from municipal civil registries. Post-discharge rehospitalizations were prospectively assessed during follow-up. Questionnaires focusing on the occurrence of MACE were sent to all living patients. Subsequently, hospital discharge letters were obtained, and treating physicians and institutions were contacted for additional information whenever necessary. ACS was defined as the clinical diagnosis of ST-segment elevation myocardial infarction (STEMI), non-STEMI or unstable angina pectoris in accordance with the guidelines of the European Society of Cardiology [17-19]. Unplanned coronary revascularization was defined as unplanned repeat PCI or coronary artery bypass grafting (CABG). The primary clinical endpoint was MACE, defined as all-cause mortality, ACS or unplanned coronary revascularization. Secondary

endpoints included acute MACE (defined as the composite of all-cause mortality or ACS) and all-cause mortality. The endpoints were adjudicated by a clinical event committee blinded for biomarker and IVUS data.

Statistical analysis

The distributions of continuous variables, including adiponectin levels and IVUS parameters, were evaluated for normality by visual examination of the histogram. Normally distributed variables are presented as mean±standard deviation (SD), while non-normally distributed variables are presented as median and interquartile range [IQR]. Adiponectin concentration was not normally distributed and was therefore ln-transformed for further analysis. Categorical variables are presented in percentages. We examined associations of adiponectin concentrations with plaque burden, plaque volume, necrotic core fraction, dense calcium fraction, fibro-fatty fraction, and fibrous tissue fraction in the imaged coronary segment by linear regression, with continuous ln-transformed adiponectin concentration as the independent variable. Furthermore, we examined the relation between adiponectin concentrations and the presence of high-risk lesions using logistic regression analyses, with continuous ln-transformed adiponectin concentration as the independent variable. Cox proportional hazards regression analyses were performed to evaluate the relationship between adiponectin concentration and MACE. Clinical variables age, gender, diabetes mellitus, hypertension, and indication for coronary angiography were considered as potential confounders and were entered into the full model. These covariates were a priori chosen based on existing literature and taking into account the number of events available. CRP was also entered into the full model, as it is the most widely investigated inflammatory marker in CAD, and has been shown to be (inversely) associated with plasma adiponectin levels [10]. When analyzing the association of adiponectin with the secondary, composite endpoint of death and ACS, and death alone, we only adjusted for age and gender because of the limited number of endpoints.

First, statistical analyses were performed in the full cohort. Then, we included interaction terms (adiponectin multiplied by indication for angiography) into the models to investigate possible effect modification by indication. Subsequently, we repeated the analyses separately in patients with SAP and patients with ACS. All data were analyzed with SPSS software (SPSS 20.0, IBM corp., Armonk, New York). All statistical tests were two-tailed and p-values <0.05 were considered statistically significant.

Results

Mean age of the patients was 61.5±11.4 years, 75.4% were men, 17.4% had diabetes mellitus, and median adiponectin concentration was 2.8 [1.9 - 4.0] μg/mL (Table 1). Coronary angiography or PCI was performed for ACS in 309 (54.2%) patients and for SAP in the remaining 261 (45.8%). Median adiponectin concentration was 2.9 [1.8 - 4.1] μg/mL in ACS patients and 2.9 [1.9 - 3.9] μg/mL in SAP patients. A total of 239 (41.9%) patients had at least 1 VH-IVUS-derived TCFA, including 69 (12.1%) patients with at least 1 VH-IVUS-derived TCFA with a plaque burden ≥70%.

In the full cohort, adiponectin levels were not associated with composition or burden of atherosclerosis on multivariable analysis (Tables 2 and 3). Adiponectin levels were not associated with MACE after adjustment for age, gender and indication for angiography (Table 4). After further multivariable adjustment, effect estimates remained non-significant (data not shown). Adiponectin levels tended to be univariably associated with acute MACE, (median [IQR] 1.16 [0.82 - 1.62] μg/mL, vs. 1.02 [0.64 - 1.38] μg/mL; HR (95% CI): 1.77 (0.96 - 3.23), p=0.069), but after further adjustment this tendency disappeared. Adiponectin levels were independently associated with occurrence of death (median [IQR] 1.48 [1.03 - 1.79] μg/mL vs. 1.02 [0.64 - 1.36] μg/mL, HR (95% CI): 2.52 (1.02 - 6.23), p=0.045).

Table 1. Baseline characteristics.

	Full cohort (n = 570)	ACS patients (n = 309)	SAP patients (n = 261)
Patient characteristics			
Age, years	61.5 ± 11.4	59.7 ± 11.9	63.6 ± 10.3
Male gender, n (%)	430 (75.4)	227 (73.5)	203 (77.8)
Hypertension, n (%)	295 (51.8)	134 (43.4)	161 (61.7)
Hypercholesterolemia, n (%)	317 (55.6)	137 (44.3)	180 (69.0)
Diabetes mellitus, n (%)	99 (17.4)	40 (12.9)	59 (22.6)
Positive family history, n (%)	293 (51.4)	140 (45.3)	153 (58.6)
Smoking, n (%)	164 (28.8)	115 (37.2)	49 (18.8)
Peripheral artery disease, n (%)	36 (6.3)	12 (3.9)	24 (9.2)
Previous myocardial infarction, n (%)	184 (32.3)	80 (25.9)	104 (39.8)
Previous PCI, n (%)	185 (32.5)	57 (18.4)	128 (49.0)
Previous CABG, n (%)	18 (3.2)	7 (2.3)	11 (4.2)
Previous stroke, n (%)	23 (4.0)	10 (3.2)	13 (5.0)
History of renal insufficiency, n (%)	32 (5.6)	13 (4.2)	19 (7.3)
History of heart failure, n (%)	19 (3.3)	6 (1.9)	13 (5.0)

Table 1 continued.

	Full cohort (n = 570)	ACS patients (n = 309)	SAP patients (n = 261)
Procedural characteristics			
PCI performed, n (%)	501 (87.9)	287 (92.9)	214 (82.0)
Indication for catheterization			
Acute coronary syndrome, n (%)	309 (54.2)	309 (100)	0 (0)
Myocardial infarction, n (%)	159 (27.9)	159 (51.5)	0 (0)
Unstable angina pectoris, n (%)	150 (26.3)	150 (48.5)	0 (0)
Stable angina pectoris, n (%)	261 (45.8)	0 (0)	261 (100)
IVUS characteristics			
Segment length, mm	44.1 [33.7 - 55.4]	43.9 [32.9 - 54.1]	44.8 [34.2 - 57.2]
Plaque burden, %	39.2 [30.0 - 46.4]	37.2 [28.0 - 45.5]	40.2 [31.8 - 47.8]
Presence lesion with MLA ≤4.0mm^2, n (%)	176 (30.9)	88 (28.7)	88 (33.7)
Presence of VH-TCFA, n (%)	239 (41.9)	140 (45.3)	99 (37.9)
Presence of VH-TCFA with PB ≥70%, n (%)	69 (12.1)	32 (10.4)	37 (14.2)
Serum biomarker concentrations			
C-reactive protein, mg/L	2.1 [0.8 - 5.3]	2.8 [1.1 - 7.0]	1.5 [0.6 - 3.1]
Adiponectin, µg/mL	2.8 [1.9 - 4.0]	2.9 [1.8 - 4.1]	2.9 [1.9 - 3.9]

ACS=acute coronary syndrome; SAP=stable angina pectoris; PCI=percutaneous coronary intervention; CABG=coronary artery bypass grafting; IVUS=intravascular ultrasound; MLA=minimal luminal area; VH-TCFA=virtual histology thin-cap fibroatheroma; PB=plaque burden. Values are mean ± SD, median [interquartile range] or n (%).

Table 2. Association of adiponectin plasma levels with segment intravascular ultrasound characteristics in the total study cohort, acute coronary syndrome and stable angina patients.

	(VH-)IVUS segment characteristics	Unadjusted model β (95% CI)[b]	P-value	Multivariable model[a] β (95% CI)[b]	P-value
Full cohort (n = 570)	Segment plaque burden	-0.40 (-2.04 – 1.23)	0.62	-0.95 (-2.74 – 0.85)	0.30
	Dense calcium fraction %	1.35 (0.27 – 2.44)	0.001	0.36 (-0.86 – 1.58)	0.56
	Necrotic core fraction %	0.43 (-0.71 – 1.58)	0.46	0.39 (-0.92 – 1.70)	0.56
	Fibrofatty tissue fraction %	-0.62 (-1.51 – 0.27)	0.17	-0.46 (-1.46 – 0.55)	0.37
	Fibrous tissue fraction %	-1.17 (-2.81 – 0.48)	0.17	-0.29 (-2.16 – 1.58)	0.76
ACS patients (n = 309)	Segment plaque burden	0.03 (-2.27 – 2.33)	0.98	-0.89 (-3.42 – 1.63)	0.49
	Dense calcium fraction %	2.53 (0.92 – 3.78)	0.001	1.10 (-0.50 – 2.70)	0.18
	Necrotic core fraction %	0.56 (-1.12 – 2.24)	0.51	0.23 (-1.69 – 2.16)	0.81
	Fibrofatty tissue fraction %	-1.47 (-2.78 – -0.15)	0.029	-0.99 (-2.49 – 0.50)	0.19
	Fibrous tissue fraction %	-1.45 (-3.78 – 0.89)	0.22	-0.35 (-3.00 – 2.30)	0.80
SAP patients (n = 261)	Segment plaque burden	-0.71 (-3.00 – 1.58)	0.54	-0.87 (-3.46 – 1.73)	0.51
	Dense calcium fraction %	0.39 (-1.25 – 2.01)	0.64	-0.41 (-2.28 – 1.47)	0.67
	Necrotic core fraction %	0.24 (-1.29 – 1.77)	0.76	0.57 (-1.20 – 2.35)	0.52
	Fibrofatty tissue fraction %	0.38 (-0.80 – 1.57)	0.52	0.01 (-1.32 – 1.34)	0.99
	Fibrous tissue fraction %	-1.01 (-3.31 – 1.30)	0.39	-0.17 (-2.82 – 2.48)	0.90

IVUS=intravascular ultrasound; CI=confidence interval of 95%; ACS=acute coronary syndrome; SAP=stable angina pectoris; CRP=C-reactive protein. [a]Adjusted for age, gender, diabetes, hypertension, and C-reactive protein (CRP). Additionally adjusted for indication for coronary angiography in the total cohort. [b]Beta per unit increase in ln-transformed adiponectin concentration.

Table 3. Association of adiponectin with presence of virtual histology intravascular ultrasound-derived high-risk lesions in the total cohort, acute coronary syndrome and stable angina patients.

	(VH-)IVUS lesion characteristics	Unadjusted model OR (95% CI)[b]	P-value	Multivariable model[a] OR (95% CI)[b]	P-value
Full cohort (n = 570)	TCFA	1.11 (0.84 – 1.49)	0.44	1.23 (0.88 – 1.71)	0.23
	TCFA PB ≥70%	0.88 (0.57 – 1.37)	0.55	0.81 (0.50 – 1.33)	0.42
	Lesion with MLA ≤4.0 mm^2	0.84 (0.62 – 1.14)	0.25	0.70 (0.49 – 1.00)	0.052
	Lesion with PB ≥70%	1.02 (0.72 – 1.44)	0.93	0.93 (0.63 – 1.39)	0.73
ACS patients (n = 309)	TCFA	0.85 (0.58 – 1.26)	0.42	0.90 (0.58 – 1.42)	0.66
	TCFA PB ≥70%	0.90 (0.57 – 1.42)	0.66	0.77 (0.37 – 1.58)	0.48
	Lesion with MLA ≤4.0 mm^2	1.13 (0.74 – 1.74)	0.57	0.87 (0.53 – 1.44)	0.59
	Lesion with PB ≥70%	1.25 (0.76 – 2.07)	0.38	1.08 (0.60 – 1.94)	0.80
SAP patients (n = 261)	TCFA	1.54 (0.99 – 2.38)	0.057	1.78 (1.06 – 3.00)	0.030
	TCFA PB ≥70%	0.86 (0.48 – 1.52)	0.60	0.87 (0.45 – 1.69)	0.68
	Lesion with MLA ≤4.0 mm^2	0.62 (0.40 – 0.97)	0.035	0.55 (0.32 – 0.93)	0.025
	Lesion with PB ≥70%	0.86 (0.54 – 1.39)	0.54	0.85 (0.49 – 1.47)	0.56

IVUS=intravascular ultrasound; OR=odds ratio; CI=confidence interval of 95%; TCFA=thin-cap fibroatheroma; PB=plaque burden; MLA=minimal luminal area; ACS=acute coronary syndrome; SAP=stable angina pectoris. [a]Adjusted for age, gender, diabetes, hypertension, and C-reactive protein (CRP). Additionally adjusted for indication for coronary angiography in the total cohort. [b]Odds ratio per unit increase in ln-transformed biomarker concentration.

Table 4. Association of adiponectin with major adverse cardiac events, secondary endpoints and death.

	Clinical outcome	Unadjusted model HR (95% CI)[b]	P-value	Adjusted for age and gender[a] HR (95% CI)[b]	P-value
Full cohort (n = 570)	MACE (n = 56)	1.28 (0.81 – 2.02)	0.29	1.19 (0.71 – 1.99)	0.52
	Acute MACE (n = 32)	1.77 (0.96 – 3.23)	0.069	1.36 (0.68 – 2.72)	0.38
	Death (n = 19)	3.36 (1.49 – 7.59)	0.004	2.52 (1.02 – 6.23)	0.045
ACS patients (n = 309)	MACE (n = 56)	1.29 (0.66 – 2.50)	0.46	1.02 (0.48 – 2.19)	0.95
	Acute MACE (n = 32)	1.75 (0.81 – 3.72)	0.14	1.40 (0.59 – 3.29)	0.44
	Death (n = 19)	2.44 (0.98 – 6.06)	0.055	1.87 (0.67 – 5.19)	0.23
SAP patients (n = 261)	MACE (n = 56)	1.30 (0.69 – 2.46)	0.42	1.43 (0.69 – 2.98)	0.34
	Acute MACE (n = 32)	1.75 (0.61 – 4.94)	0.29	1.33 (0.41 – 4.28)	0.64
	Death (n = 19)	8.15 (1.49 – 44.68)	0.016	8.48 (0.92 – 78.03)	0.058

HR=hazard ratio; CI=confidence interval of 95%; MACE=major adverse cardiac events; ACS=acute coronary syndrome; SAP=stable angina pectoris; Acute MACE=composite of death or acute coronary syndrome (secondary endpoints). [a]Additionally adjusted for indication for coronary angiography in the total cohort. [b]Hazard ratio per unit increase in ln-transformed biomarker concentration.

Signs of interactions between adiponectin and indication for angiography were present for associations with TCFA (p for interaction 0.050 (univariable) and 0.029 (multivariable)), with lesions with MLA ≤4.0mm^2 (p for interaction 0.058 (univariable) and 0.10 (multivariable)), and with fibrofatty tissue fraction (p for interaction 0.042 (univariable) and 0.082

(multivariable)). The remaining interaction terms were not significant (data not shown).
In patients with SAP, adiponectin levels were associated with the presence of VH-IVUS-derived TCFA lesions (median [IQR] 1.16 [0.72 - 1.48] μg/mL vs. 0.95 [0.62 - 1.30] μg/mL; OR (95% CI) per 1 unit increase in ln-transformed-adiponectin: 1.78 (1.06 - 3.00), p=0.030) (Table 3). Furthermore, adiponectin levels were inversely associated with presence of lesions with MLA ≤4.0 mm^2 (median [IQR] 0.95 [0.49 - 1.30] μg/mL vs. 1.06 [0.69 - 1.41] μg/mL; OR (95% CI): 0.55 (0.32 - 0.93), p=0.025) (Table 3). Finally, adiponectin levels were associated with death (median [IQR] 1.62 [1.32 - 1.84] μg/mL vs. 1.02 [0.64 - 1.36] μg/mL; HR (95% CI): 8.15 (1.49 - 44.68)). After adjustment for age and gender, the HR remained similar in magnitude, although statistical significance was lost (HR (95% CI): 8.48 (0.92 - 78.03), p=0.058).

In patients with ACS, no associations were present between adiponectin and composition or burden of atherosclerosis. Although no associations were present with MACE or acute MACE, a tendency toward a univariable association with death was present (median [IQR] 1.39 [0.90 - 1.86] μg/mL vs. 1.01 [0.60 - 1.38] μg/mL; HR (95% CI): 2.44 (0.98 - 6.06), p=0.055). After adjustment for age and gender, statistical significance was lost (HR (95% CI): 1.87 (0.67 - 5.19), p=0.23).

Given the positive associations we found between adiponectin and death, we performed a post-hoc analysis to explore whether a synergistic effect of adiponectin and TCFA was present on death. For this purpose, we entered interaction terms into the models that consisted of adiponectin multiplied by presence of TCFA lesions. However, no effect modification could be demonstrated (interaction terms were not significant).

Discussion

To our best knowledge, this is the largest study that correlates circulating adiponectin with *in-vivo* measurements of coronary atherosclerosis using VH-IVUS in patients with known coronary disease. We found that in the full cohort, adiponectin levels were associated with death during 1-year follow-up, but not with VH-IVUS measures of atherosclerosis. In patients with SAP, adiponectin levels were positively associated with presence of VH-IVUS-derived TCFA lesions and were inversely associated with presence of lesions with MLA ≤ 4.0 mm^2; while the association with death was borderline significant. In ACS patients we only found a

tendency toward an association with death during follow-up.

Fundamental experiments, animal models and human studies on vascular function in subjects free of symptomatic cardiovascular disease have all demonstrated associations of adiponectin with vasoprotective mechanisms, including insulin-sensitizing characteristics and anti-oxidative and anti-inflammatory properties [4-6,8,10]. In line with this, higher levels of adiponectin have been linked to decreased prevalence of CAD in healthy individuals and have demonstrated an inverse association with risk of myocardial infarction [20,21]. However, in patients with manifested CAD, adiponectin seems to play a different role. When elevated in patients with symptomatic CAD, this adipocytokine becomes associated with an increased risk of cardiovascular events; a phenomenon that has been described under the term "reverse epidemiology" [22-25]. To explain these conflicting findings, it has been proposed that increased adiponectin levels reflect a compensatory and vasculoprotective mechanism [25]. Specifically, in conditions characterized by a marked systemic pro-inflammatory state and endothelial dysfunction, adiponectin levels increase as an attempt to counter-regulate or compensate for this systemic inflammation. Consequently, the protective effects of adiponectin are superseded by the underlying disease [25].

In a cohort of 981 patients with stable ischemic heart disease, with average follow-up of 7.1 years, an association was found between higher adiponectin and adverse cardiovascular events (death, heart failure), but after adjustment for cardiac disease severity, the association was no longer statically significant [24]. Another cohort with median follow-up of 2.5 years found that higher adiponectin levels were associated with future cardiovascular death or nonfatal myocardial infarction in SAP patients (n=1130), but found no association in ACS patients (n=760) [22]. Our results, demonstrating an association of adiponectin with death in SAP patients, comply with these findings. The lack of statistical significance for this association in ACS patients in our study may, in part, have been caused by a limited number of clinical events. Moreover, pathophysiological differences may possibly have contributed to the difference we found between SAP and ACS. While in SAP patients, atherosclerosis appears to be a slowly progressing disorder, in ACS patients, coronary plaque rupture may be present, and the latter is accompanied by the production of tissue factor and other homeostatic factors that increase the risk of thrombosis [3]. Plasma adiponectin levels have been inversely correlated with markers of platelet activation [26,27]. This might have possibly influenced the association between adiponectin and clinical outcome in these patients.

Adipose tissue produces both pro- and anti-inflammatory adipocytokines [10], and

adiponectin has shown *in vitro* and *in vivo* anti-inflammatory effects [28]. However, little is known about the clinical significance of adiponectin for coronary plaque stability *in vivo*. Only a few studies have been performed on this topic, all of which at the University of Kobe, Japan. Sample size of these studies was modest. In a randomized trial of 54 patients with type 2 diabetes and stable angina, treated with pioglitazone, adiponectin was found to be associated with a reduction of necrotic core components as assessed by VH-IVUS [29]. A case control study of 63 ACS and 43 non-ACS patients showed that serum adiponectin was inversely associated with necrotic core evaluated by VH-IVUS in both culprit and non-culprit lesions in patients with ACS, but not in those with stable angina [30]. In 50 men with stable CAD, low plasma adiponectin was associated with presence of TCFA [31]. Altogether, these studies point toward an inverse association of adiponectin with plaque vulnerability. In contrast, in our study, we found a positive association of adiponectin with VH-IVUS TCFA in SAP patients. This finding is in line with the association of adiponectin with death, as well as the association of VH-IVUS TCFA with adverse outcome which we demonstrated earlier [12]. However, the exact mechanism behind the positive association between adiponectin and VH-IVUS-derived TCFA lesions warrants further investigation. With regard to the discrepancy between our study and the Japanese ones, differences in study population and sample size could have played a part. Ethnic differences in adiponectin levels are of particular interest in this context. The Mediators of Atherosclerosis in South Asians Living in America (MASALA) study and the Multi-Ethnic Study of Atherosclerosis (MESA) have shown that adiponectin levels are lowest in persons from South Asian or Chinese descent compared to other ethnic groups [32]. Moreover, polymorphisms in the adiponectin gene have been found to be associated with adiponectin levels [33]. Some of these polymorphisms have also shown associations with insulin resistance, metabolic syndrome and the onset of CAD [32-34]. Finally, while we found a positive association of adiponectin with VH-IVUS TCFA, we could not demonstrate such an association with necrotic core fraction. This seeming discrepancy may be explained by the fact that these measures reflect somewhat different aspects of atherosclerosis. Size of necrotic core fraction alone may not be able to fully capture the properties of rupture-prone plaques; the definition of VH-IVUS TCFA on its part incorporates additional plaque properties, such as confluence of the necrotic core and direct contact of the necrotic core with the lumen.

Some limitations of this study need to be acknowledged. The spatial resolution of VH-IVUS (200mm) is insufficient to exactly replicate histopathological definitions of a thin fibrous cap (<65mm) [13]. Therefore, VH-IVUS tends to over-estimate the number of TCFA

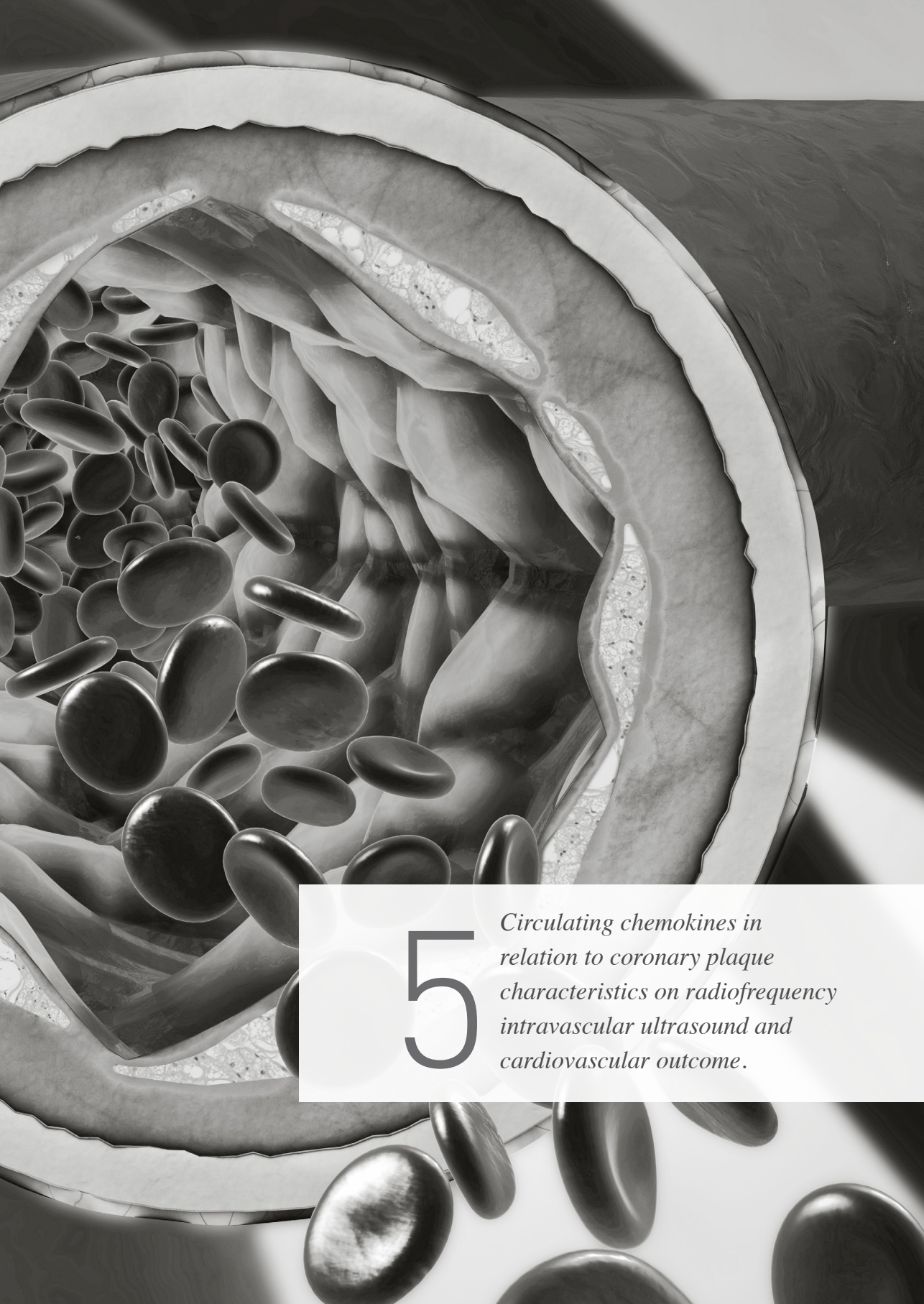
lesions. Nevertheless, the presence of VH-IVUS-detected TCFA lesions carries prognostic information [12] and is therefore clinically relevant. Furthermore, VH-IVUS imaging was performed in a prespecified single target segment of a single non-culprit coronary artery [35]. This approach was chosen because previous studies have demonstrated that such segments reflect larger coronary disease burden and are associated with subsequent cardiac events [12,36]. Finally, adiponectin was associated with mortality, but the number of deaths in our dataset was small.

In conclusion, in the full cohort, adiponectin levels were associated with death but not with VH-IVUS measures of atherosclerosis. In SAP patients, adiponectin levels were associated with VH-IVUS derived TCFA lesions, while the association with death was borderline significant. Altogether, a substantial role for adiponectin in plaque vulnerability remains unconfirmed and warrants investigation by other, large studies.

References

1. Newby D.E. Triggering of acute myocardial infarction: beyond the vulnerable plaque. Heart 2010; 96: 1247-1251.
2. Narula J. et al. Arithmetic of vulnerable plaques for noninvasive imaging. Nat Clin Pract Cardiovasc Med 2008; 5 Suppl 2: S2-10.
3. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med 1999; 340: 115-126.
4. Shimada K. et al. Adiponectin and atherosclerotic disease. Clin Chim Acta 2004; 344: 1-12.
5. Barseghian A. et al. Adiponectin and vulnerable atherosclerotic plaques. J Am Coll Cardiol 2011; 57: 761-770.
6. Matsuzawa Y. Adiponectin: Identification, physiology and clinical relevance in metabolic and vascular disease. Atheroscler Suppl 2005; 6: 7-14.
7. Kubota N. et al. Disruption of adiponectin causes insulin resistance and neointimal formation. J Biol Chem 2002; 277: 25863-25866.
8. Szmitko P.E. et al. Adiponectin and cardiovascular disease: state of the art? Am J Physiol Heart Circ Physiol 2007; 292: H1655-1663.
9. Sattar N. et al. Adiponectin and coronary heart disease: a prospective study and meta-analysis. Circulation 2006; 114: 623-629. Erratum in: Circulation 2007; 115: e325.
10. Ouchi N. and Walsh K. Adiponectin as an anti-inflammatory factor. Clin Chim Acta 2007; 380: 24-30.
11. de Boer S.P. et al. Relation of genetic profile and novel circulating biomarkers with coronary plaque phenotype as determined by intravascular ultrasound: rationale and design of the ATHEROREMO-IVUS study. EuroIntervention 2014; 10: 953-960.
12. Cheng J.M. et al. In vivo detection of high-risk coronary plaques by radiofrequency intravascular ultrasound and cardiovascular outcome: results of the ATHEROREMO-IVUS study. Eur Heart J 2014; 35: 639-47.
13. Garcia-Garcia H.M. et al. Tissue characterisation using intravascular radiofrequency data analysis: recommendations for acquisition, analysis, interpretation and reporting. EuroIntervention 2009; 5: 177-189.
14. Nair A. et al. Automated coronary plaque characterisation with intravascular ultrasound backscatter: ex vivo validation. EuroIntervention 2007; 3: 113-120.
15. García-García H.M. et al. Tissue characterisation using intravascular radiofrequency data analysis: recommendations for acquisition, analysis, interpretation and reporting. EuroIntervention 2009; 5:177-189.
16. Rodriguez-Granillo G.A. et al. In vivo intravascular ultrasound-derived thin-cap fibroatheroma detection using ultrasound radiofrequency data analysis. J Am Coll Cardiol 2005; 46: 2038-2042.
17. Erhardt L. et al. Task force on the management of chest pain. Task force on the management of chest pain. Eur Heart J 2002; 23: 1153-76.
18. Hamm C.W. et al. ESC Committee for Practice Guidelines. ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: The Task Force for the management of acute coronary syndromes (ACS) in patients presenting without persistent ST-segment elevation of the European Society of Cardiology (ESC). Eur Heart J 2011; 32: 2999-3054.
19. Van de Werf F. et al. ESC Committee for Practice Guidelines (CPG). Management of acute myocardial infarction in patients presenting with persistent ST-segment elevation: the Task Force on the Management of ST-Segment Elevation Acute Myocardial Infarction of the European Society of Cardiology. Eur Heart J 2008; 29: 2909-2945.
20. Laughlin G.A. et al. Association of adiponectin with coronary heart disease and mortality: the Rancho Bernardo study. Am J Epidemiol 2007; 165: 164-174.

21. Pischon T. et al. Plasma adiponectin levels and risk of myocardial infarction in men. JAMA 2004; 291: 1730-1737.
22. Schnabel R. et al. Association of adiponectin with adverse outcome in coronary artery disease patients: results from the AtheroGene study. Eur Heart J 2008; 29: 649-657.
23. Forsblom C. et al. FinnDiane Study Group. Serum adiponectin concentration is a positive predictor of all-cause and cardiovascular mortality in type 1 diabetes. J Intern Med 2011; 270: 346-355.
24. Beatty A.L. et al. Adiponectin is associated with increased mortality and heart failure in patients with stable ischemic heart disease: data from the Heart and Soul Study. Atherosclerosis 2012; 220: 587-592.
25. Rathmann W. and Herder C. Adiponectin and cardiovascular mortality: evidence for "reverse epidemiology". Horm Metab Res 2007; 39: 1-2.
26. Shoji T. et al. Platelet activation is associated with hypoadiponectinemia and carotid atherosclerosis. Atherosclerosis 2006; 188: 190-195.
27. Bigalke B. et al. High plasma levels of adipocytokines are associated with platelet activation in patients with coronary artery disease. Platelets 2010; 21: 11-19.
28. Van de Voorde J. et al. Adipocytokines in relation to cardiovascular disease. Metabolism 2013; 62: 1513-1521.
29. Ogasawara D. et al. Pioglitazone reduces the necrotic-core component in coronary plaque in association with enhanced plasma adiponectin in patients with type 2 diabetes mellitus. Circ J 2009; 73: 343-351.
30. Otake H. et al. Relation between plasma adiponectin, high-sensitivity C-reactive protein, and coronary plaque components in patients with acute coronary syndrome. Am J Cardiol 2008; 101: 1-7.
31. Sawada T. et al. Low plasma adiponectin levels are associated with presence of thin-cap fibroatheroma in men with stable coronary artery disease. Int J Cardiol 2010; 142: 250-256.
32. Shah A.D. et al. Less favorable body composition and adipokines in south asians compared to other U.S. ethnic groups: results from the MASALA and MESA studies. Int J Obes (Lond) 2016; 40: 639-645.
33. Yang W.S. and Chuang L.M. Human genetics of adiponectin in the metabolic syndrome. J Mol Med (Berl) 2006; 84: 112-121.
34. Ohashi K. et al. Adiponectin I164T mutation is associated with the metabolic syndrome and coronary artery disease. J Am Coll Cardiol 2004; 43: 1195-1200.
35. Garcia-Garcia H.M. et al. Imaging of coronary atherosclerosis: intravascular ultrasound. Eur Heart J, 2010; 31: 2456-2469.
36. Nicholls S.J. et al. Intravascular ultrasound-derived measures of coronary atherosclerotic plaque burden and clinical outcome. J Am Coll Cardiol 2010; 55: 2399-2407.



5

Circulating chemokines in relation to coronary plaque characteristics on radiofrequency intravascular ultrasound and cardiovascular outcome.

5

Circulating chemokines in relation to coronary plaque characteristics on radiofrequency intravascular ultrasound and cardiovascular outcome.

Cheng JM, Oemrawsingh RM, Akkerhuis KM, Garcia-Garcia HM, de Boer SP, Battes LC, Buljubasic N, Lenzen MJ, de Jaegere PP, van Geuns RJ, Serruys PW, Kardys I, Boersma E.

Biomarkers 2014; 19(7): 611-619. doi: 10.3109/1354750X.2014.957725.

Abstract

Rationale: To investigate relations of several circulating chemokines with extent and phenotype of coronary atherosclerosis and with 1-year clinical outcome.

Methods & Results: Intravascular ultrasound virtual histology (IVUS-VH) imaging of a coronary artery was performed in 581 patients. Monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1a (MIP-1a), MIP-1b and regulated upon activation normal T cell expressed and secreted (RANTES) were measured in plasma. Higher MCP-1, MIP-1a and lower RANTES were associated with coronary plaque burden. Higher MCP-1, MIP-1a and lower RANTES were associated with the presence of IVUS-VH-derived thin-cap fibroatheroma lesions. RANTES was associated with major adverse cardiac events.

Conclusions: RANTES is a promising biomarker that is inversely associated with coronary plaque burden and vulnerability, as well as with death and acute coronary syndrome.

Introduction

Inflammation has been recognized as an important contributing factor in all phases of atherosclerosis [1-3]. In particular, inflammation is believed to play a crucial role in the development and rupture of vulnerable plaques, resulting in major cardiovascular problems such as myocardial infarction and stroke [1-3]. Circulating inflammatory biomarkers may potentially improve prognostication of patients with atherosclerotic cardiovascular disease [4]. Chemokines are involved in the recruitment of various leukocytes, such as monocytes, macrophages and T lymphocytes, into the atherosclerotic plaque [5,6]. Monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1a (MIP-1a), MIP-1b and regulated upon activation normal T cell expressed and secreted (RANTES) are typical C-C motif chemokines that have been studied extensively [5,6]. Several studies have shown that these chemokines have an important role throughout the entire atherosclerotic process from atherogenesis to plaque destabilization [5,6]. However, their clinical utility as biomarker remains unclear [5,6]. Furthermore, prospective data on associations of these biomarkers with in-vivo measurements of extensiveness, phenotype and vulnerability of coronary atherosclerosis is currently lacking. This study aims to evaluate the usefulness of MCP-1, MIP-1a, MIP-1b and RANTES by investigating their relations with intravascular ultrasound virtual histology (IVUS-VH)-derived measures of coronary plaque burden, quantity of necrotic core, and presence of thin-cap fibroatheroma (TCFA) lesions, and by investigating their prognostic value for major adverse cardiac events.

Methods

Study population

The design of The European Collaborative Project on Inflammation and Vascular Wall Remodeling in Atherosclerosis – Intravascular Ultrasound (ATHEROREMO-IVUS) study has been described in detail elsewhere [7,8]. In brief, 581 patients who underwent diagnostic coronary angiography or percutaneous coronary intervention (PCI) for acute coronary syndrome (ACS) or stable angina pectoris (SAP) have been included between 2008 and 2011 in the Erasmus MC, Rotterdam, the Netherlands (Supplementary Figure 1). The ATHEROREMO-IVUS study was approved by the medical ethics committee of the Erasmus

MC. The study was performed in accordance with the criteria described in the declaration of Helsinki. Written informed consent was obtained from all included patients. This study is registered in ClinicalTrials.gov, number NCT01789411.

Data collection

Baseline characteristics of all patients were collected prospectively by trained research physicians. These physicians reviewed the medical charts of the patients at the time of inclusion in the study, and extracted variables regarding demographics, medical history, cardiovascular risk factors and procedural characteristics. Medical history and cardiovascular risk factors are a routine part of clinical patient assessment at the department of Cardiology. Thus, presence of diabetes mellitus, hypertension, hypercholesterolemia, history of renal insufficiency and history of heart failure were defined as a clinical diagnosis of these conditions as reported by the treating physician in the medical chart. Smoking was defined as current smoking, reported by the patient. Procedural characteristics were prospectively extracted from the catheterization report.

Biomarkers

Blood samples were drawn from the arterial sheath prior to the diagnostic coronary angiography or PCI procedure. The blood samples were transported to the clinical laboratory of Erasmus MC for further processing and storage at temperature of -80 ^{o}C within 2h after blood collection. MCP-1, MIP-1a, MIP-1b and RANTES were measured in the stored EDTA-plasma samples (n=570) using a validated multiplex assay (Custom Human Map, Myriad RBM, Austin, TX).

Intravascular ultrasound

Following the standard coronary angiography or PCI procedure, IVUS data were acquired in a non-culprit coronary vessel. Selection of the non-culprit vessel was predefined in the study protocol. The order of preference for selection of the non-culprit vessel was: (1) left anterior descending (LAD) artery; (2) right coronary artery (RCA); 3. left circumflex (LCX) artery. All IVUS data were acquired with the Volcano s5/s5i Imaging System (Volcano Corp., San Diego, CA) using a Volcano Eagle Eye Gold IVUS catheter (20 MHz). An automatic pullback system was used with a standard pull back speed of 0.5mm per second. The IVUS images were analyzed offline by an independent core laboratory (Cardialysis BV, Rotterdam,

the Netherlands) that had no knowledge of clinical data. The IVUS gray-scale and IVUS radiofrequency analyses, also known as IVUS virtual histology, were performed using pcVH 2.1 and qVH (Volcano Corp., San Diego, CA) software. The external elastic membrane and luminal borders were contoured for each frame (median inter-slice distance, 0.40mm). Extent and phenotype of the atherosclerotic plaque were assessed. Plaque burden was defined as plaque and media cross-sectional area divided by external elastic membrane cross-sectional area and is presented as a percentage. The composition of the atherosclerotic plaque was characterized into four different tissue types: fibrous, fibrofatty, dense calcium and necrotic core [9]. A coronary lesion was defined as a segment with a plaque burden of more than 40% in at least three consecutive frames (Supplementary Figure 2). A TCFA lesion on IVUS-VH was defined as a lesion with presence of >10% confluent necrotic core in direct contact with the lumen (Figure 1) [10,11]. TCFA lesions with a plaque burden of at least 70% were classified as large TCFA lesions.

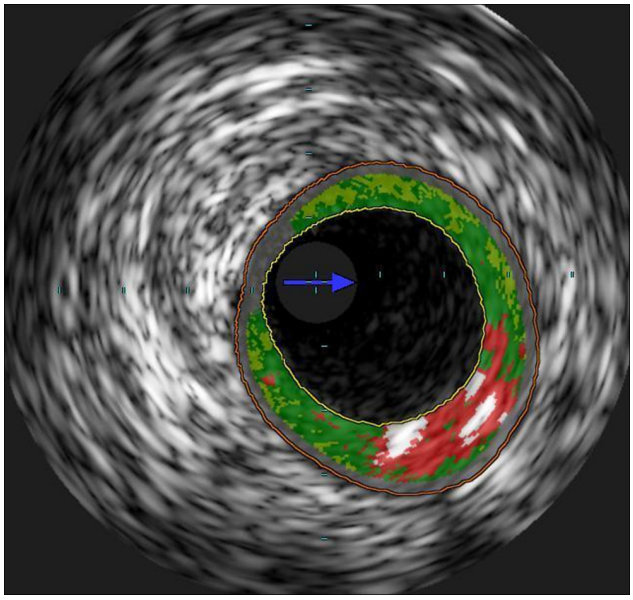


Figure 1. Thin-cap fibroatheroma lesion on intravascular ultrasound virtual histology. Thin-cap fibroatheroma lesion on intravascular ultrasound virtual histology is defined as a lesion with presence of >10% confluent necrotic core (red) in direct contact with the lumen. White indicates dense calcium, light green indicates fibrofatty tissue, dark green indicates fibrous tissue.

Study endpoints

In this study, follow-up started at inclusion and lasted up to 1 year. Post-discharge survival status was obtained from municipal civil registries. Post-discharge rehospitalizations were prospectively assessed during follow-up. Questionnaires focusing on the occurrence of major adverse cardiac events (MACE) were sent to all living patients. Treating physicians and institutions were contacted for additional information whenever necessary. ACS was defined as the clinical diagnosis of ST segment elevation myocardial infarction (STEMI), non-STEMI or unstable angina pectoris in accordance with the guidelines of the European Society of Cardiology [12-14]. Unplanned coronary revascularization was defined as unplanned repeat PCI or coronary artery bypass grafting (CABG). All events were adjudicated as related to a coronary site that was treated during the index procedure (culprit lesion related event) or as related to the coronary site that was not treated during the index procedure (non-culprit lesion related event). Events that were related to both the culprit lesion and a non-culprit site (e.g. revascularization of multiple vessels with CABG) were classified into both categories. When information was not sufficient to classify an event as either culprit lesion related or non-culprit lesion related, the event was classified as indeterminate.

The primary endpoint was MACE, defined as non-culprit lesion related or indeterminate all-cause mortality, ACS or unplanned coronary revascularization. The secondary endpoint was defined as the composite of non-culprit lesion related or indeterminate all-cause mortality or ACS. Definite culprit lesion related events were excluded from the primary and secondary endpoints, because the pathophysiology of culprit lesions related events (e.g. in-stent restenosis or in-stent thrombosis) differs from our primary research focus on spontaneous plaque rupture leading to unanticipated, spontaneous MACE. The endpoints were adjudicated by a clinical event committee that had no knowledge of biomarkers and IVUS data.

Statistical analysis

The distributions of the continuous variables, including biomarker levels and the IVUS parameters, were tested for normality by visual examination of the histogram. Normally distributed continuous variables are presented as mean±standard deviation (SD), while non-normally distributed continuous variables are presented as median and interquartile range (IQR). MCP-1, MIP-1a, MIP-1b and RANTES concentrations were not normally distributed and were therefore ln-tranformed for further analysis. Categorical variables are presented in percentages. We examined associations of biomarker concentrations with plaque burden and

necrotic core fraction in the imaged coronary segment. Specifically, we calculated means of plaque burden and necrotic core fraction according to tertiles of biomarker concentration. To test for trends, we used linear regression analyses with continuous ln-transformed biomarker concentrations as the independent variable. The final results are presented as b (per SD increase in ln-transformed biomarker concentration) with 95% confidence interval (95% CI). Furthermore, we have examined the relation between biomarker concentrations and the presence of IVUS-VH derived TCFA lesions using logistic regression analyses with continuous ln-tranformed biomarker concentration as the independent variable. The final results are presented as odds ratio (OR) per SD increase in ln-transformed biomarker concentration with 95% CI.

Patients lost to follow-up were considered at risk until the date of last contact, at which time-point they were censored. Cumulative event rates were estimated according to the Kaplan–Meier method. Cumulative Kaplan–Meier event curves were compared by log-rank test. Cox proportional hazards regression analyses were performed to evaluate the relationship between biomarker concentration and clinical endpoints. Biomarkers that were significantly associated with occurrence of MACE in univariable analysis were further evaluated in multivariable analyses. The variables age, gender, diabetes mellitus, hypertension, hypercholesterolemia, smoking, statin use, history of MI and indication for coronary angiography were considered as potential confounders and were entered into the full model. These covariates were a priori chosen, taking into account the number of events available. Subsequently, C-reactive protein (CRP) was also entered into the model to evaluate whether the associations between biomarkers and MACE were independent of CRP concentration. The final results are presented as hazard ratio (HR) per SD increase in ln-transformed biomarker concentration with 95% CI.

All statistical analyses were primarily performed in the overall study population. Heterogeneity in effect estimates between patients with ACS and patients with stable angina were examined using the Z-test for heterogeneity. If there was no heterogeneity, conclusions were based on the effect estimates belonging to the total study population. If there was significant heterogeneity between patients admitted with and without ACS, conclusions were based on effect estimates of the separate groups.

All data were analyzed with SPSS software (SPSS 20.0, IBM corp., Armonk, NY). All statistical tests were two-tailed and p values < 0.05 were considered statistically significant.

Results

Baseline characteristics

Mean age of the patients was 61.5±11.4 years, 75.4% were men and 17.4% had diabetes mellitus (Table 1). Coronary angiography or PCI was performed for various indications: 159 (27.9%) patients had an acute myocardial infarction, 150 (26.3%) patients had unstable angina pectoris and 261 (45.8%) patients had stable angina pectoris. Some patients had biomarker concentrations beneath the lowest detection limit of the assay, which especially pertains to MIP-1a (measurable in 84% of patients). The median length of the imaged coronary segment was 44.1 [33.7 – 55.4] mm. On basis of radiofrequency IVUS, a total of 239 (41.9%) patients had at least 1 IVUS-VH-derived TCFA, including 69 (12.1%) patients with at least 1 IVUS-VH-derived TCFA with a plaque burden ≥70%.

Table 1. Baseline characteristics.

	Total (n = 570)	ACS patients (n = 309)	SAP patients (n = 261)
Patient characteristics			
Age, years	61.5 ± 11.4	59.7 ± 11.9	63.6 ± 10.3
Male gender, n (%)	430 (75.4)	227 (73.5)	203 (77.8)
Hypertension, n (%)	295 (51.8)	134 (43.4)	161 (61.7)
Hypercholesterolemia, n (%)	317 (55.6)	137 (44.3)	180 (69.0)
Diabetes mellitus, n (%)	99 (17.4)	40 (12.9)	59 (22.6)
Positive family history, n (%)	293 (51.4)	140 (45.3)	153 (58.6)
Smoking, n (%)	164 (28.8)	115 (37.2)	49 (18.8)
Peripheral artery disease, n (%)	36 (6.3)	12 (3.9)	24 (9.2)
Previous myocardial infarction, n (%)	184 (32.3)	80 (25.9)	104 (39.8)
Previous PCI, n (%)	185 (32.5)	57 (18.4)	128 (49.0)
Previous CABG, n (%)	18 (3.2)	7 (2.3)	11 (4.2)
Previous stroke, n (%)	23 (4.0)	10 (3.2)	13 (5.0)
History of renal insufficiency, n (%)	32 (5.6)	13 (4.2)	19 (7.3)
History of heart failure, n (%)	19 (3.3)	6 (1.9)	13 (5.0)
C-reactive protein, mg/L	2.1 [0.8–5.3]	2.8 [1.1–7.0]	1.5 [0.6–3.1]
Statin use, n (%)	359 (63.0)	146 (47.2)	213 (81.6)
Procedural characteristics			
PCI performed, n (%)	501 (87.9)	287 (92.9)	214 (82.0)
Indication for catheterization			
Acute coronary syndrome, n (%)	309 (54.2)	309 (100)	0 (0)
Myocardial infarction, n (%)	159 (27.9)	159 (51.5)	0 (0)
Unstable angina pectoris, n (%)	150 (26.3)	150 (48.5)	0 (0)
Stable angina pectoris, n (%)	261 (45.8)	0 (0)	261 (100)

Table 1 continued.

	Total (n = 570)	ACS patients (n = 309)	SAP patients (n = 261)
Procedural characteristics			
IVUS segment characteristics			
Imaged coronary artery			
Left anterior descending, n (%)	204 (35.8)	117 (37.9)	87 (33.3)
Left circumflex, n (%)	190 (33.3)	107 (34.6)	83 (31.8)
Right coronary artery, n (%)	176 (30.9)	85 (27.5)	91 (34.9)
Segment length, mm	44.1 [33.7-55.4]	43.9 [32.9-54.1]	44.8 [34.2-57.2]
Presence of at least 1 TCFA, n (%)	239 (41.9)	140 (45.3)	99 (37.9)
Presence of at least 1 TCFA with PB ≥70%, n (%)	69 (12.1)	32 (10.4)	37 (14.2)
Serum biomarker concentrations			
MCP-1, pg/ml[a]	91 [70–122]	92 [70–133]	88 [71–111]
MIP-1a, pg/ml[b]	16.0 [12.0–21.9]	15.0 [12.0–21.9]	17.0 [12.0–21.9]
MIP-1b, pg/ml[a]	123 [92–165]	130 [95–179]	114 [89–146]
RANTES, ng/ml[c]	11.0 [6.4–19.0]	14.0 [7.6–23.0]	9.1 [5.0–14.3]

ACS=acute coronary syndrome; CABG=coronary artery bypass grafting; IVUS=intravascular ultrasound; MCP-1, monocyte chemoattractant protein-1; MIP-1a, macrophage inflammatory protein-1a; MIP-1b, macrophage inflammatory protein-1b; MLA=minimal luminal area; PB=plaque burden; PCI=percutaneous coronary intervention; RANTES, Regulated upon Activation Normal T cell Expressed and Secreted; SAP=stable angina pectoris; TCFA=thin-cap fibroatheroma. Values are mean ± SD, median [interquartile range] or n (%). [a]Measurable in >99% of patients; below limit of detection in <1% of patients. [b]Measurable in 84% of patients; below limit of detection in 16% of patients. [c]Measurable in all patients.

Associations with coronary atherosclerosis

In patients who were admitted with stable angina pectoris, higher plasma MCP-1 concentrations were associated with higher coronary plaque burden (per SD increase of ln-transformed MCP-1: β=2.56, 95% CI 0.91 - 4.21, p=0.002) and a higher fraction of plaque consisting of necrotic core (per SD increase of ln-transformed MCP-1: β=1.14, 95% CI 0.02 - 2.25, p=0.045) (Table 2). Higher MCP-1 concentrations also seemed to be associated with the presence of IVUS-VH derived TCFA lesions (OR per SD increase in ln-transformed MCP-1 1.90, 95% CI 1.00 - 3.61, p=0.052) in patients who were admitted with stable angina pectoris (Table 3).

Higher MIP-1a concentrations were associated with higher plaque burden (per SD increase of ln-transformed MIP-1a: β=1.66, 95% CI 0.72 - 2.61, p=0.001), higher necrotic core fraction (per SD increase of ln-transformed MIP-1a: β=0.89, 95% CI 0.23 - 1.55, p=0.008) and with the presence of IVUS-VH derived TCFA lesions with plaque burden ≥70% (OR per SD increase in ln-transformed MIP-1a 1.75, 95% CI 1.09 - 2.81, p=0.021) in the total study population.

Table 2. Associations with plaque burden and necrotic core fraction in imaged coronary segment.

	Total study population (n = 570)				ACS patients (n = 309)				SAP patients (n = 261)				P-value for heterogeneity
	Tertile 1[a]	Tertile 2[a]	Tertile 3[a]	P	Tertile 1[a]	Tertile 2[a]	Tertile 3[a]	P	Tertile 1[a]	Tertile 2[a]	Tertile 3[a]	P	
Mean values of plaque burden (%)													
MCP-1	38.0±11.0	37.8±11.3	38.9±12.4	0.46	38.4±11.9	35.7±11.0	36.9±12.5	0.49	37.7±9.9	40.2±10.7	41.0±12.3	0.002	0.004
MIP-1α	36.9±10.8	37.8± 9.8	39.0±11.9	0.001	35.1±10.6	36.5±10.1	39.3±12.2	0.001	38.8±10.9	39.8±9.0	38.6±11.7	0.38	0.10
MIP-1β	36.7±11.2	39.0± 11.5	39.0±11.8	0.31	36.5±12.2	38.6±11.4	36.0±11.8	0.84	37.3±10.1	39.5±11.9	42.1±10.8	0.015	0.071
RANTES	39.5±10.9	37.7± 12.2	37.5±11.4	0.089	38.8±11.4	37.3±12.0	34.9±11.8	0.025	39.4±10.3	38.3±12.0	41.2±10.8	0.32	0.022
Mean values of necrotic core fraction (%)													
MCP-1	21.3±8.1	21.3± 7.3	21.6±8.8	0.84	22.6±8.4	21.1±8.2	21.5±9.2	0.32	19.6±7.3	21.6±6.5	21.9±8.1	0.045	0.027
MIP-1α	21.1±7.6	21.6± 7.2	21.5±8.7	0.008	21.7±7.9	21.0±7.4	23.0±9.3	0.009	20.1±7.2	22.4±6.7	19.9±7.7	0.33	0.27
MIP-1β	21.4±8.0	21.4± 7.5	21.4±8.7	0.76	21.9±8.1	21.3±8.0	22.0±9.6	0.84	20.8±7.8	21.5±6.1	20.9±8.1	0.91	0.83
RANTES	21.8±7.3	21.1± 9.1	21.4±7.8	0.53	22.8±8.1	21.6±9.1	20.8±8.5	0.17	21.0±6.4	20.4±8.3	21.8±7.4	0.81	0.24

ACS=acute coronary syndrome; MCP-1=monocyte chemoattractant protein-1; MIP-1a=macrophage inflammatory protein-1a; MIP-1b=macrophage inflammatory protein-1b; RANTES=Regulated upon Activation Normal T cell Expressed and Secreted; SAP=stable angina pectoris. [a]Tertiles of biomarker levels. P-values were obtained with linear regression analyses with continuous ln-transformed biomarker concentration as independent variable.

In patients who were admitted with ACS, lower RANTES concentrations were associated with higher plaque burden (per SD increase of ln-transformed RANTES: β=1.57, 95% CI -2.94 - -0.20, p=0.025) (Figure 2). Furthermore, lower RANTES concentrations also seemed to be associated with the presence of IVUS-VH derived TCFA lesions with plaque burden ≥70% in the overall patient population (OR per SD increase in ln-transformed RANTES 0.76, 95% CI 0.57 - 1.02, p=0.067).

Major adverse cardiac events

Vital status was acquired for 569 (99.8%) patients. Response rate of the questionnaires that were sent to all living patients was 92.3%. After 1 year of follow-up, 56 patients had at least one event (Supplementary Table 1). A total of 11 patients had a definite culprit lesion related event, while 27 patients had a definite non-culprit lesion related event. Another 18 patients had an event that could not be judged to be either culprit lesion related or non-culprit lesion related and were therefore classified as having an indeterminate event. The cumulative Kaplan–Meier incidences of the 30-d, 6-month and 1-year composite of non-culprit lesion related or indeterminate death, ACS or unplanned coronary revascularization were 0.7%, 4.7%, and 7.9%, respectively. The cumulative Kaplan–Meier incidences of the 30-d, 6-month and 1-year composite of non-culprit lesion related or indeterminate death or ACS were 0.7%, 3.2%, and 4.9%, respectively.

Table 3. Associations with presence of intravascular ultrasound virtual histology-derived thin-cap fibroatheroma lesions.

	Total study population (n = 570)		ACS patients (n = 309)		SAP patients (n = 261)		P-value for heterogeneity
	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P	
Presence of at least 1 thin-cap fibroatheroma							
MCP-1	1.03 (0.74–1.45)	0.85	0.77 (0.51–1.17)	0.22	1.90 (1.00–3.61)	0.052	0.022
MIP-1α	0.87 (0.63–1.21)	0.42	0.94 (0.61–1.42)	0.75	0.83 (0.49–1.39)	0.47	0.72
MIP-1β	1.16 (0.85–1.60)	0.36	1.18 (0.79–1.76)	0.42	0.97 (0.55–1.70)	0.91	0.69
RANTES	0.97 (0.80–1.18)	0.75	0.87 (0.66–1.15)	0.33	0.98 (0.72–1.33)	0.90	0.57
Presence of at least 1 thin-cap fibroatheroma with plaque burden ≥70%							
MCP-1	1.23 (0.75–2.04)	0.41	0.94 (0.48–1.83)	0.86	2.16 (0.95–4.93)	0.067	0.12
MIP-1α	1.75 (1.09–2.81)	0.021	2.15 (1.13–4.09)	0.020	1.29 (0.63–2.66)	0.49	0.30
MIP-1β	0.89 (0.54–1.47)	0.66	0.91 (0.47–1.78)	0.79	1.01 (0.46–2.20)	0.98	0.85
RANTES	0.76 (0.57–1.02)	0.067	0.73 (0.47–1.15)	0.17	0.84 (0.55–1.28)	0.41	0.67

ACS=acute coronary syndrome; MCP-1=monocyte chemoattractant protein-1; MIP-1a=macrophage inflammatory protein-1a; MIP-1b=macrophage inflammatory protein-1b; RANTES=Regulated upon Activation Normal T cell Expressed and Secreted; SAP=stable angina pectoris. Odds ratios are per standard deviation increase in ln-transformed biomarker concentration.

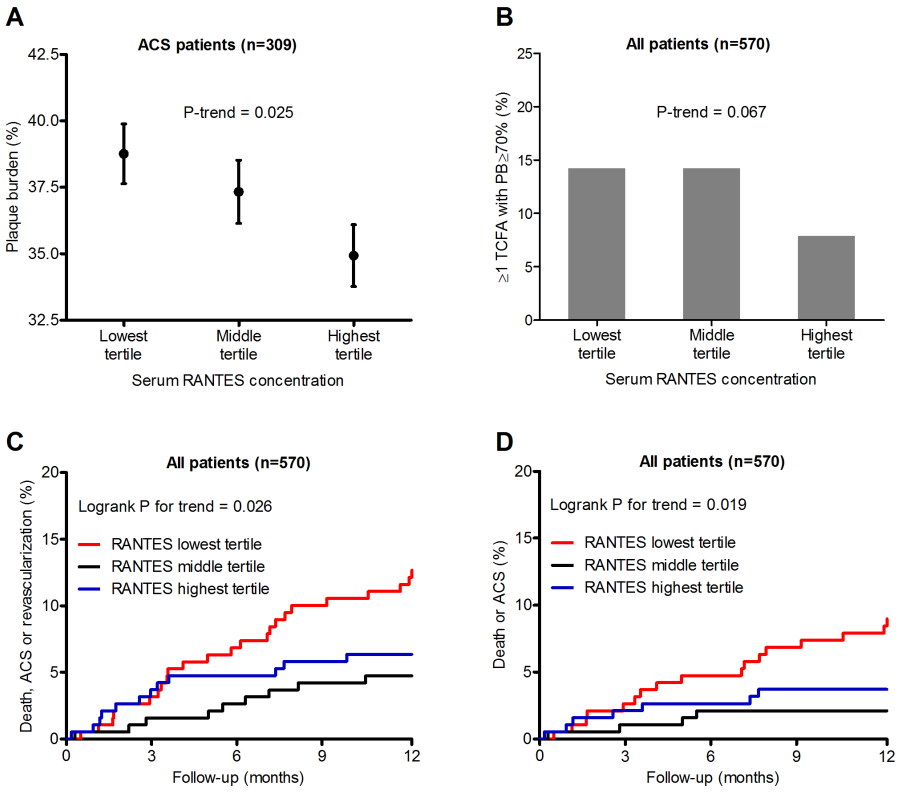


Figure 2. Associations of circulating RANTES concentrations with coronary atherosclerosis and clinical outcome.

ACS=acute coronary syndrome; PB=plaque burden; RANTES=Regulated upon Activation Normal T cell Expressed and Secreted; TCFA=thin-cap fibroatheroma. (A) Association with intravascular ultrasound-derived measures of coronary plaque burden in patients admitted with acute coronary syndrome. (B) Association with presence of TCFA with plaque burden ≥70% as assessed by intravascular ultrasound virtual histolgy. (C) Association with occurrence of non-culprit lesion related and indeterminate death, acute coronary syndrome or coronary revascularization. The lowest RANTES tertile was associated with the highest event rate (lowest tertile versus middle tertile p=0.006; lowest tertile versus highest tertile p=0.042; middle tertile versus highest tertile p=0.50; logrank p for trend=0.026). (D) Association with occurrence of non-culprit lesion related and indeterminate death or acute coronary syndrome. The lowest RANTES tertile was associated with the highest event rate (lowest tertile versus middle tertile p=0.004; lowest tertile versus highest tertile p=0.039; middle tertile versus highest tertile p=0.86; logrank p for trend=0.019).

Associations with non-culprit lesion related and indeterminate events

In univariable analysis, RANTES (HR per SD increase of ln-transformed RANTES 0.67, 95% CI 0.50 - 0.89, p=0.005) was associated with occurrence of the primary endpoint of non-culprit lesion related and indeterminate MACE during follow-up (Table 4, Figure 2). There was no heterogeneity in the HR estimate between ACS patients and patients with stable angina

(heterogeneity p=0.39). RANTES (HR per SD increase of ln-transformed RANTES 0.64, 95% CI 0.45 - 0.91, p=0.013) was also significantly associated with the composite of non-culprit lesion related and indeterminate death or ACS only. After adjustment for conventional cardiovascular risk factors in multivariable analysis, RANTES remained independently predictive for non-culprit lesion related and indeterminate MACE (HR per SD increase of ln-transformed RANTES 0.69, 95% CI 0.52 - 0.93, p=0.016) and for non-culprit lesion related and indeterminate death or ACS only (HR per SD increase of ln-transformed RANTES 0.60, 95% CI 0.41 - 0.88, p=0.010) (Table 5). RANTES also remained independently associated with MACE (HR per SD increase of ln-transformed RANTES 0.69, 95% CI 0.51 - 0.93, p=0.014) and the composite of death or ACS only (HR per SD increase of ln-transformed RANTES 0.59, 95% CI 0.40 - 0.88, p=0.010) after additional adjustment for baseline CRP levels. Subgroup analysis showed that the inverse association between RANTES level and MACE was present in all patient subgroups (Figure 3). There was no significant heterogeneity in the HR estimate between the evaluated patient subgroups.

Table 4. Associations with non-culprit lesion related and indeterminate major adverse cardiac events.

	Total study population (n = 570)		ACS patients (n = 309)		SAP patients (n = 261)		P-value for heterogeneity
	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P	
Major adverse cardiac events (primary endpoint)							
MCP-1	0.87 (0.64–1.18)	0.37	0.81 (0.55–1.20)	0.29	1.00 (0.61–1.65)	1.00	0.51
MIP-1α	1.13 (0.85–1.49)	0.40	1.16 (0.82–1.66)	0.40	1.06 (0.69–1.64)	0.80	0.74
MIP-1β	1.00 (0.74–1.34)	0.99	1.15 (0.82–1.62)	0.42	0.82 (0.50–1.34)	0.42	0.26
RANTES	0.67 (0.50–0.89)	0.005	0.77 (0.50–1.18)	0.23	0.59 (0.40–0.88)	0.009	0.39
Composite of death or acute coronary syndrome (secondary endpoint)							
MCP-1	0.73 (0.48–1.09)	0.12	0.74 (0.47–1.16)	0.19	0.69 (0.31–1.53)	0.36	0.88
MIP-1α	1.11 (0.77–1.58)	0.58	1.12 (0.73–1.70)	0.61	1.11 (0.59–2.09)	0.74	0.99
MIP-1β	1.11 (0.78–1.57)	0.57	1.34 (0.98–1.84)	0.071	0.48 (0.24–0.98)	0.043	0.010
RANTES	0.64 (0.45–0.91)	0.013	0.58 (0.36–0.94)	0.028	0.62 (0.35–1.10)	0.10	0.86

ACS=acute coronary syndrome; MCP-1=monocyte chemoattractant protein-1; MIP-1a=macrophage inflammatory protein-1a; MIP-1b=macrophage inflammatory protein-1b; RANTES=Regulated upon Activation Normal T cell Expressed and Secreted; SAP=stable angina pectoris. Hazard ratios are per standard deviation increase in ln-transformed biomarker concentration.

Table 5. Multivariable analysis on non-culprit lesion related and indeterminate major adverse cardiac events.

	Adjusted for age and gender		Adjusted for age, gender and indication for angiography		Adjusted for conventional risk factors and indication for angiography[a]		Adjusted for conventional risk factors, indication for angiography and CRP[a]	
	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
Major adverse cardiac events (primary endpoint)								
RANTES	0.72 (0.54–0.96)	0.024	0.71 (0.53–0.95)	0.023	0.69 (0.52–0.93)	0.016	0.69 (0.51–0.93)	0.014
Composite of death or acute coronary syndrome (secondary endpoint)								
RANTES	0.69 (0.48–0.99)	0.046	0.64 (0.44–0.93)	0.021	0.60 (0.41–0.88)	0.010	0.59 (0.40–0.88)	0.010

CRP=C-reactive protein; RANTES=regulated upon activation normal T cell expressed and secreted. [a]Conventional risk factors include: age, gender, diabetes mellitus, hypertension, hypercholesterolemia, smoking, statin use and history of myocardial infarction. Hazard ratios are per standard deviation increase in ln-transformed biomarker concentration.

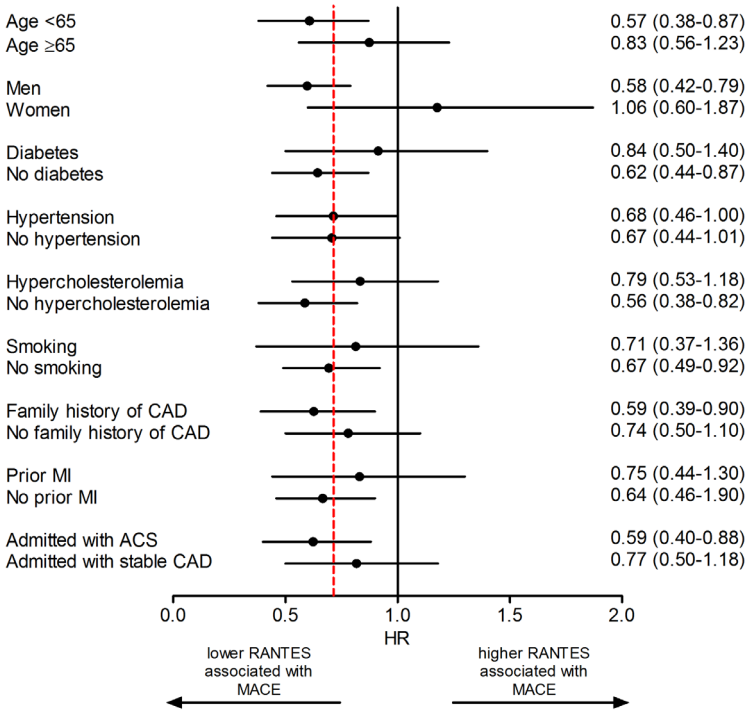


Figure 3. Association between RANTES level and major adverse cardiac events stratified by patient subgroups. ACS=acute coronary syndrome; CAD=coronary artery disease; HR=hazard ratio; MI=myocardial infarction; RANTES=Regulated upon Activation Normal T cell Expressed and Secreted. Hazard ratios (95% confidence intervals) are per standard deviation increase in ln-transformed RANTES concentration. Red dotted line indicates the hazard ratio estimate in the total study population.

Discussion

This study investigated the relations of circulating chemokine concentrations with extensiveness of coronary atherosclerosis, amount of necrotic core, the presence of IVUS-VH derived TCFA lesions and occurrence of future MACE in patients who underwent coronary angiography for ACS or stable angina pectoris. To our best knowledge, this is the first study that correlates circulating chemokines with in-vivo measurements of coronary atherosclerosis using IVUS-VH. Higher plasma MCP-1, MIP-1a, and lower RANTES concentrations were all associated with higher coronary plaque burden and more advanced plaque phenotypes as determined by IVUS-VH (Figure 4). However, only RANTES was found to be independently predictive for the occurrence of MACE, particularly of death and ACS.

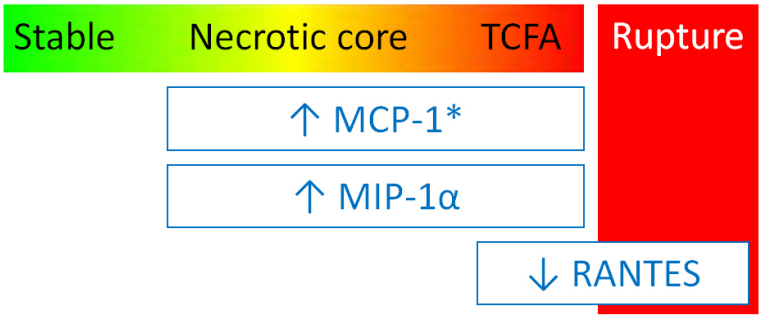


Figure 4. Circulating chemokine concentrations as biomarkers for phenotype of coronary atherosclerosis and risk of plaque rupture.

MCP-1=monocyte chemoattractant protein-1; MIP-1a=macrophage inflammatory protein-1a; RANTES=Regulated upon Activation Normal T cell Expressed and Secreted; TCFA=thin-cap fibroatheroma. Hypothesized model based on findings in this study. Plasma MCP-1, MIP-1a and RANTES concentrations were all associated with coronary plaque burden and different plaque phenotypes. RANTES was associated with major adverse cardiac events that were most probably caused by plaque rupture. *Only for patients with stable angina pectoris. ↑ indicates higher biomarker concentrations. ↓ indicates lower biomarker concentrations.

Chemokines are small cytokines that have the ability to induce directed chemotaxis of nearby leukocytes. MCP-1, MIP-1a, MIP-1b and RANTES belong to the C-C motif chemokine ligand (CCL) family and are also known as CCL2, CCL3, CCL4 and CCL5, respectively [5,6]. Pathologic studies have shown that these chemokines are highly expressed in atherosclerotic plaques [15-17]. Animal studies have shown that these chemokines are actively involved in atherogenesis and plaque destabilization [5,6]. Furthermore, several epidemiological studies have indicated that serum or plasma levels of MCP-1, MIP-1a, MIP-1b and RANTES may predict future cardiac events [5]. However, their clinical utility as biomarker for cardiovascular risk stratification remains unclear [5,6]. We sought to further elucidate the correlations of circulating chemokine concentrations with in-vivo measurements of extensiveness, phenotype and vulnerability of coronary atherosclerosis by using IVUS-VH.

Grayscale IVUS allows for in-vivo measurements of coronary plaque burden. Additionally, radiofrequency IVUS allows for differentiation of the composition of the atherosclerotic plaque and is therefore also known as IVUS-VH [10]. Necrotic core is often found in the more advanced and rupture-prone plaques [18]. The Providing Regional Observations to Study Predictors of Events in the Coronary Tree (PROSPECT) study has demonstrated that TCFA lesions as determined by IVUS-VH are associated with MACE [19]. The strong and independent associations (adjusted HR's ranging from 1.79 to 3.35) of IVUS-VH-derived TCFA with MACE emphasize its biological importance [18-20]. However, there are several reasons why IVUS is currently not suitable for use as diagnostic and prognostic

tool in the overall population of patients with coronary artery disease [19]. Its invasiveness is probably the most important limitation in this respect. Therefore, circulating biomarkers may have an important role in cardiovascular risk assessment.

In our study, lower plasma RANTES concentrations were independently associated with adverse outcomes during 1 year of follow-up. The association was independent of CRP. Its association with acute cardiac events (death or ACS; HR 0.59) seemed to be even stronger than with all MACE (death, ACS or unplanned coronary revascularization; HR 0.69). This may indicate that RANTES is especially predictive for plaque rupture rather than plaque growth. Our finding that low serum RANTES concentrations, rather than high, are associated with adverse coronary events may seem counterintuitive, since animal studies have shown that RANTES and its receptor are actively involved in atherogenesis and that RANTES was found to be highly expressed within atheromatous lesions [6,21]. However, the inverse associations of RANTES may be explained by increased deposition of RANTES on the vascular endothelium, resulting in lower free-circulating serum concentrations [22,23]. The inverse associations of RANTES are also consistent with observations from previous studies. A large case–control study reported that serum RANTES levels were lower in coronary heart disease patients compared with age- and gender-matched controls [23]. Another study reported that low plasma RANTES levels were independently associated with cardiac mortality in 389 male patients who underwent coronary angiography [22]. Such an association was not found in a population-based case-cohort study that included 363 individuals with incident coronary events and 1908 non-cases [24].

We found that higher plasma MCP-1 concentrations were associated with higher coronary plaque burden in patients who were admitted with stable angina pectoris. These findings are in line with a previous study that measured MCP-1 concentrations in blood from the coronary sinus and found that these levels were associated with the extent of coronary atherosclerosis as assessed on the coronary angiogram [25]. Although we observed that high MCP-1 concentrations were associated with a more advanced plaque phenotype (i.e. higher necrotic core fraction) and with the presence of IVUS-VH derived TCFA lesions, MCP-1 was not predictive for future events. Previous epidemiological studies have shown that the ability of MCP-1 to predict subclinical coronary artery disease is somewhat disappointing, but that MCP-1 may have some value in predicting cardiovascular events in patients with overt coronary artery disease [5]. For example, a previous study found that MCP-1 was independently associated with the composite of death or myocardial infarction in a large

cohort of 4244 patients with ACS [26]. This study also demonstrated that high MCP-1 values at 4 months after the initial ACS were still predictive for long-term mortality afterwards. A major difference with our study is that both culprit lesion related and non-culprit lesion related events were included in their study endpoints, while definite culprit lesion related events were excluded from our study endpoints. Furthermore, we may have lacked statistical power to detect the previously reported association.

MIP-1a has been studied less extensively. We found that MIP-1a was associated with coronary plaque burden, necrotic core fraction and with the presence of large TCFA lesions on IVUS-VH. However, we did not observe a correlation between MIP-1a concentration and occurrence of MACE. Another study, however, found that MIP-1a was predictive for recurrent ACS in a relatively small cohort of 54 patients with unstable angina pectoris [27]. Further research is required to elucidate the role of MIP-1a in patients with coronary artery disease.

Conclusions

Higher circulating MCP-1, MIP-1a, and lower RANTES concentrations were associated with a higher extent, a more advanced phenotype and a higher vulnerability of coronary atherosclerosis. Such associations were not present for MIP-1b. In addition, RANTES was independently associated with occurrence of MACE, particularly of death and ACS. Its prognostic value was similar in patients with and without ACS. Its inverse associations are consistent with observations from previous studies and may be explained by increased deposition of RANTES on the endothelium, resulting in lower free-circulating concentrations. The findings in this study demonstrate that RANTES may be a useful biomarker for assessment of cardiovascular risk. Further research on the incremental prognostic value of RANTES over established clinical covariates in large, prospective studies is warranted.

References

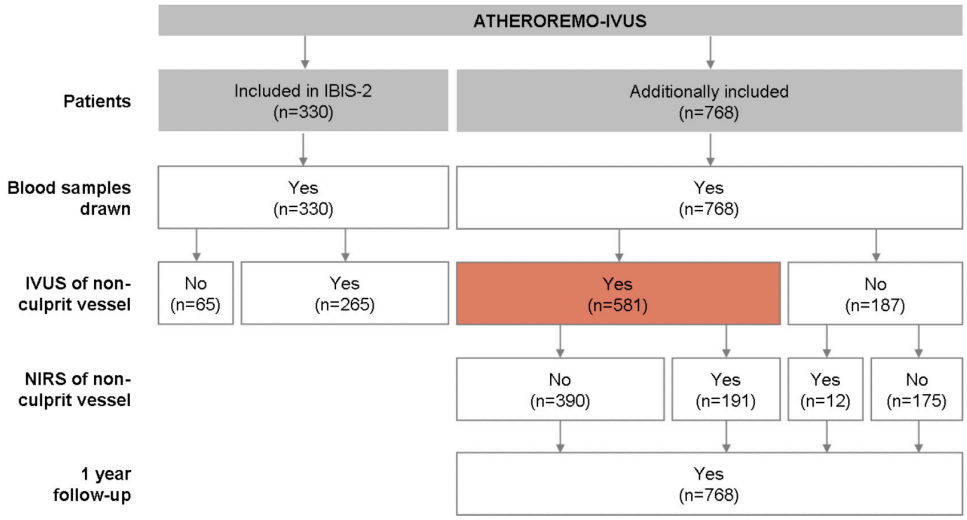
1. Armstrong E.J. et al. Inflammatory biomarkers in acute coronary syndromes: part I: introduction and cytokines. Circulation 2006; 113: e72–75.
2. Hansson G.K. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 2005; 352: 1685–1695.
3. Libby P. Inflammation and atherosclerosis. Circulation 2002; 105: 1135–1143.
4. Wykrzykowska J.J. et al. Differential protein biomarker expression and their time-course in patients with a spectrum of stable and unstable coronary syndromes in the Integrated Biomarker and Imaging Study-1 (IBIS-1). Int J Cardiol 2011; 149: 10–16.
5. Aukrust P. et al. Chemokines and cardiovascular risk. Arterioscler Thromb Vasc Biol 2008; 28: 1909–1919.
6. Weber C. et al. Chemokines: key regulators of mononuclear cell recruitment in atherosclerotic vascular disease. Arterioscler Thromb Vasc Biol 2004; 24: 1997–2008.
7. Cheng J.M. et al. In-vivo detection of high risk coronary plaques by intravascular ultrasound and cardiovascular outcome: results of the ATHEROREMO-IVUS study. Eur Heart J 2014; 35: 639–647.
8. De Boer S.P.M. et al. Relation of genetic profile and novel circulating biomarkers with coronary plaque phenotype as determined by intravascular ultrasound: rationale and design of the ATHEROREMO-IVUS study. EuroIntervention 2013; doi: 10.4244/EIJY13M08_01.
9. Nair A. et al. Automated coronary plaque characterisation with intravascular ultrasound backscatter: ex vivo validation. EuroIntervention 2007; 3: 113–120.
10. Garcia-Garcia H.M. et al. Tissue characterisation using intravascular radiofrequency data analysis: recommendations for acquisition, analysis, interpretation and reporting. EuroIntervention 2009; 5: 177–189.
11. Rodriguez-Granillo G.A. et al. In vivo intravascular ultrasound-derived thin-cap fibroatheroma detection using ultrasound radiofrequency data analysis. J Am Coll Cardiol 2005; 46: 2038–2042.
12. Erhardt L. et al. Task force on the management of chest pain. Eur Heart J 2002; 23: 1153–1176.
13. Hamm C.W. et al. ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: the Task Force for the management of acute coronary syndromes (ACS) in patients presenting without persistent ST-segment elevation of the European Society of Cardiology (ESC). Eur Heart J 2011, 32: 2999–3054.
14. Van de Werf F. et al. Management of acute myocardial infarction in patients presenting with persistent Stsegment elevation: the Task force on the management of ST-segment elevation acute myocardial infarction of the European society of cardiology. Eur Heart J 2008; 29: 2909–2945.
15. Nelken N.A. et al. Monocyte chemoattractant protein-1 in human atheromatous plaques. J Clin Invest 1991; 88: 1121–1127.
16. Pattison J.M. et al. RANTES chemokine expression in transplant-associated accelerated atherosclerosis. J Heart Lung Transplant 1996; 15: 1194–1199.
17. Wilcox J.N. et al. Local expression of inflammatory cytokines in human atherosclerotic plaques. J Atheroscler Thromb 1994; 1: S10–13.
18. Virmani R. et al. Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. Arterioscler Thromb Vasc Biol 2000; 20: 1262–1275.
19. Stone G.W. et al. A prospective naturalhistory study of coronary atherosclerosis. N Engl J Med 2011; 364: 226–235.
20. Calvert P.A. et al. Association between IVUS findings and adverse outcomes in patients with coronary artery

disease: the VIVA (VH-IVUS in Vulnerable Atherosclerosis) Study. JACC Cardiovasc Imag 2011; 4: 894–901.
21. Veillard N.R. et al. Antagonism of RANTES receptors reduces atherosclerotic plaque formation in mice. Circ Res 2004; 94: 253–261.
22. Cavusoglu E. et al. Low plasma RANTES levels are an independent predictor of cardiac mortality in patients referred for coronary angiography. Arterioscler Thromb Vasc Biol 2007; 27: 929–935.
23. Rothenbacher D. et al. Differential expression of chemokines, risk of stable coronary heart disease, and correlation with established cardiovascular risk markers. Arterioscler Thromb Vasc Biol 2006; 26: 194–199.
24. Herder C. et al. RANTES/CCL5 and risk for coronary events: results from the MONICA/KORA Augsburg casecohort, Athero-Express and CARDIoGRAM studies. PLoS One 2011; 6: e25734.
25. Serrano-Martinez M. et al. Monocyte chemoattractant protein-1 concentration in coronary sinus blood and severity of coronary disease. Circulation 2003; 108: e75.
26. de Lemos J.A. et al. Serial measurement of monocyte chemoattractant protein-1 after acute coronary syndromes: results from the A to Z trial. J Am Coll Cardiol 2007; 50: 2117–2124.
27. de Jager S.C. et al. CCL3 (MIP-1 alpha) levels are elevated during acute coronary syndromes and show strong prognostic power for future ischemic events. J Mol Cell Cardiol 2008; 45: 446–452.

Supplementary files

Supplementary Table 1. Patients with major adverse cardiac events.

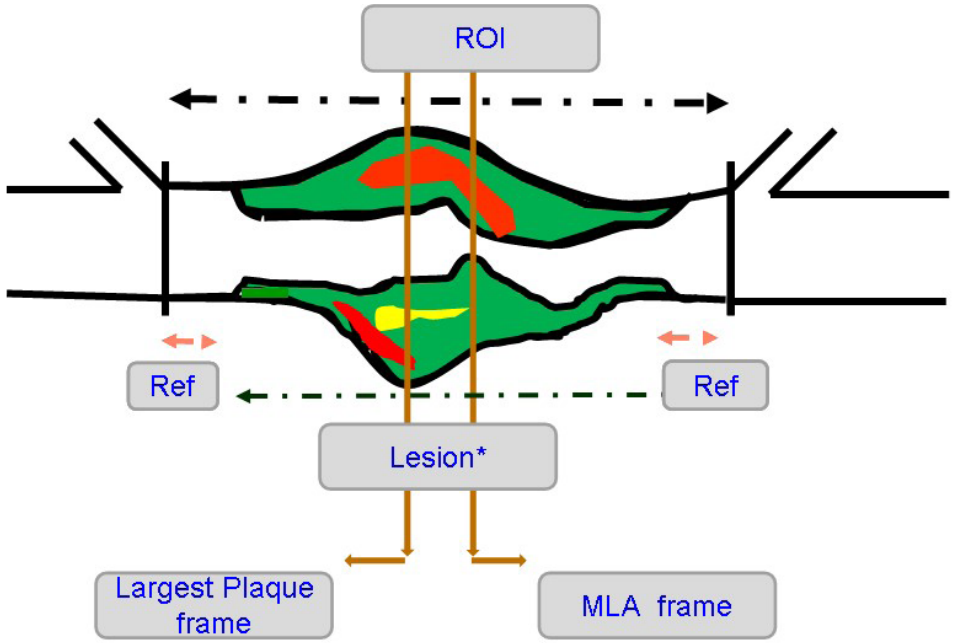
	Culprit lesion related events, n	Non-culprit lesion related events, n	Indeterminate events, n	Non-culprit lesion related and indeterminate events combined, n	All events, n
Composite of major adverse cardiac events	11	27	18	45	56
Death from any cause, n	1	1	16	17	18
Definite cardiac or unexplained sudden death, n	1	1	6	7	8
Acute coronary syndrome, n	3	9	2	11	14
Myocardial infarction, n	2	3	2	5	7
Elective coronary revascularization, n	7	17	0	17	24
Composite of death or acute coronary syndrome, n	4	10	18	28	32



Supplementary Figure 1. Patient inclusion.

IBIS-2=Integrated Biomarker and Imaging Study-2; IVUS=intravascular ultrasound; NIRS=near-infrared spectroscopy.

Patients were included in the current study analyses when the following criteria were met: 1. not participating in the IBIS-2 trial; 2. IVUS of a non-culprit coronary artery was performed; and 3. plasma samples were available for biomarker measurements. In patients who were additionally included at Erasmus MC (n=768), IVUS of a non-culprit coronary artery was performed in 581 patients (red shaded). In these patients, blood samples were available in 570 patients.



Supplementary Figure 2. Methodology for detection of lesion and reference segments.
MLA=minimal luminal area; REF=reference segments; ROI=region of interest.

6

Circulating cytokines in relation to the extent and composition of coronary atherosclerosis: Results from the ATHEROREMO-IVUS study.

6 Circulating cytokines in relation to the extent and composition of coronary atherosclerosis: Results from the ATHEROREMO-IVUS study.

Battes LC, Cheng JM, Oemrawsingh RM, Boersma E, Garcia-Garcia HM, de Boer SP, Buljubasic N, Mieghem NA, Regar E, Geuns RJ, Serruys PW, Akkerhuis KM, Kardys I.

Atherosclerosis 2014; 236(1): 18-24. doi: 10.1016/j.atherosclerosis.2014.06.010.

Abstract

Rationale: We investigated whether concentrations of TNF-α, TNF-β, TNF-receptor 2, interferon-γ, IL-6, IL-8, IL-10 and IL-18 are associated with extent and composition of coronary atherosclerosis determined by grayscale and virtual histology (VH)- intravascular ultrasound (IVUS).

Methods & Results: Between 2008 and 2011, IVUS(-VH) imaging of a non-culprit coronary artery was performed in 581 patients (stable angina pectoris (SAP), n=261; acute coronary syndrome (ACS), n=309) undergoing coronary angiography from the ATHEROREMO-IVUS study. Plaque burden, presence of VH-IVUS-derived thin-cap fibroatheroma (TCFA) lesions, and presence of VH-TCFA lesions with plaque burden ≥70% were assessed. Blood samples for cytokine measurement were drawn from the arterial sheath prior to the angiography procedure. We applied linear and logistic regression. TNF-α levels were positively associated with plaque burden (beta (β) (95% CI): 4.45 (0.99 - 7.91), for highest vs lowest TNF-α tertile) and presence of VH-TCFA lesions (odds ratio (OR) (95% CI) 2.30 (1.17 - 4.52),

highest vs lowest TNF-α tertile) in SAP patients. Overall, an inverse association was found between IL-10 concentration and plaque burden (β (95% CI): -1.52 (-2.49 - -0.55), per ln (pg/mL) IL-10) as well as IL-10 and VH-TCFA lesions with plaque burden ≥70% (OR: 0.31 (0.12 - 0.80), highest vs lowest IL-10 tertile). These effects did not reach statistical significance in the separate SAP and ACS groups.

Conclusions: Higher circulating TNF-α was associated with higher plaque burden and VH-TCFA lesions in SAP patients. Lower circulating IL-10 was associated with higher plaque burden and large VH-TCFA lesions. These in-vivo findings suggest a role for these cytokines in extent and vulnerability of atherosclerosis.

Introduction

Inflammation is known to play a major role in atherosclerosis [1-3]. The development of atherosclerosis includes, among others, expression of adhesion molecules by inflamed endothelium, migration of leukocytes into the intima, uptake of modified lipoprotein particles, and formation of lipid-laden macrophages [4]. During the evolution of atherosclerotic lesions, T-lymphocytes join the macrophages in the intima [4]. This T-cell infiltrate produces proinflammatory cytokines (including tumor necrosis factors (TNFs), interferons (IFNs), and interleukins (ILs), but may also stimulate a T helper cell type 2 (Th2) response which can promote anti-inflammatory actions (and cytokines such as IL-10 and transforming growth factor β) [2,5]. This dual role of cytokines is believed to control the subsequent development and destabilization of atherosclerotic plaques in coronary (among other) arteries [6], potentially leading to plaque rupture or erosion and ultimately resulting in adverse clinical events such as myocardial infarction or sudden cardiac death [7].

While previous research has provided ample insights into the signaling cascades of cytokines and their roles in the pathogenesis of atherosclerosis, studies on the associations of cytokines with in-vivo determined extent and particularly composition of coronary atherosclerosis are currently scarce. Cytokines are located both inside the affected vessel walls and in the circulation [8]. We hypothesize that circulating cytokines are associated with in-vivo measures of plaque burden and features of plaque vulnerability, and consequently may be useful for clinical risk stratification with regard to cardiovascular outcome.

The aim of this study is to examine the associations of the cytokines TNF-α, TNF-β, interferon-γ (IFN-γ), IL-6, IL-8, IL-10 and IL-18 and of circulating TNF receptor 2 (TNF R2) with the extent and composition of coronary atherosclerosis as determined in-vivo by intravascular ultrasound (IVUS) and IVUS-virtual histology (IVUS-VH), in a non-culprit vessel in patients undergoing coronary angiography.

Methods

Study population

The design of The European Collaborative Project on Inflammation and Vascular Wall Remodeling in Atherosclerosis - Intravascular Ultrasound (ATHEROREMO-IVUS) study has been described elsewhere [9]. In brief, 581 patients who underwent diagnostic coronary angiography or percutaneous coronary intervention (PCI) for acute coronary syndrome(ACS; n=309) or stable angina pectoris (SAP; n=261) have been included from November 2008 to January 2011 in the Erasmus MC, Rotterdam, the Netherlands. Intravascular ultrasound (IVUS) of a non-culprit coronary artery was performed subsequent to angiography. The ATHEROREMO-IVUS study has been approved by the human research ethics committee of Erasmus MC, Rotterdam, The Netherlands. Written informed consent was obtained from all included patients and the study protocol conforms to the ethical guidelines of the Declaration of Helsinki.

Biomarkers

Blood samples were drawn from the arterial sheath prior to the diagnostic coronary angiography or PCI procedure, and were available in 570 patients for the current study. The blood samples were transported to the clinical laboratory of Erasmus MC for further processing and storage at a temperature of -80 ^{o}C within 2h after blood collection.

C-reactive protein (CRP) was measured in serum samples using an immunoturbidimetric high sensitivity assay (Roche Diagnostics Ltd., Rotkreuz, Switzerland) on the Cobas 8000 modular analyzer platform (Roche Diagnostics Ltd., Rotkreuz, Switzerland). These analyses were performed in the clinical laboratory of Erasmus MC.

Frozen EDTA-plasma samples were transported under controlled conditions (at a temperature of -80 ^{o}C) to Myriad RBM, Austin, Texas, USA, where the concentrations

of TNF-α, TNF-β, TNF R2, IFN-γ, IL-6, IL-8, IL-10 and IL-18 were determined using a validated multiplex assay (Custom Human Map, Myriad RBM, Austin, Texas, USA). While TNF-α, TNF R2, IL-6, and IL-8 were determined in the full cohort of 570 patients, TNF-β, IFN-γ, IL-10 and IL-18 were determined in a random subset of 473 patients. This difference in numbers resulted from batch-wise handling of the samples in combination with an update of the composition of the multiplex assay by the manufacturer in-between two batches. None of the biomarker laboratories had knowledge of clinical or intracoronary imaging data.

Intravascular ultrasound

Following the standard coronary angiography or PCI procedure, IVUS data were acquired in a non-culprit, non-treated, coronary vessel, without significant luminal narrowing. The order of preference for selection of the non-culprit vessel was: 1. Left anterior descending (LAD) artery; 2. Right coronary artery (RCA); 3. Left circumflex (LCX) artery. All IVUS data were acquired with the Volcano s5/s5i Imaging System (Volcano Corp., San Diego, CA, USA) using a Volcano Eagle Eye Gold IVUS catheter (20 MHz). An automatic pullback system was used with a standard pull back speed of 0.5mm per second. The IVUS images were analyzed offline by an independent core laboratory (Cardialysis BV, Rotterdam, the Netherlands) that had no knowledge of clinical or biomarker data. The IVUS gray-scale and IVUS radiofrequency analyses, also known as IVUS virtual histology (IVUS-VH), were performed using pcVH 2.1 and qVH (Volcano Corp., San Diego, CA, USA) software. The external elastic membrane and luminal borders were contoured for each frame (median interslice distance, 0.40mm). Extent and phenotype of the atherosclerotic plaque were assessed.

Plaque burden was defined as the plaque and media cross-sectional area divided by the external elastic membrane cross-sectional area and is presented as a percentage. A coronary lesion was defined as a segment with a plaque burden of 40% in at least three consecutive frames [9]. Using IVUS-VH, the composition of the atherosclerotic plaque was characterized into 4 different types: fibrous, fibro-fatty, dense calcium and necrotic core [10]. A VH-IVUS-derived thin-cap fibroatheroma (TCFA) lesion was defined as a lesion with presence of >10% confluent necrotic core in direct contact with the lumen [11].

Statistical analysis

Categorical variables are presented in percentages. The distributions of continuous variables,

including biomarker levels and IVUS parameters, were examined for normality by visual inspection of the histogram and calculation of the skewness coefficient. Normally distributed continuous variables are presented as mean±standard deviation (SD), while non-normally distributed continuous variables are presented as median and interquartile range [IQR]. For reasons of uniformity, all biomarkers are presented as median [IQR].

In further analyses, biomarker concentrations were examined both as continuous and as categorical variables (the latter by dividing the variables into tertiles). Biomarkers with a non-normal distribution were ln-transformed. Biomarkers in which the concentrations were too low to detect in more than 20% of the patients, were not examined as continuous variables. They were examined as tertiles, or else as dichotomous variables (measurable vs not measurable).

To take into account possible effect modification by indication for coronary angiography, we performed all analyses separately in patients with SAP and patients with ACS. We also present the results for the full cohort, in order to evaluate the effect of higher statistical power in those cases where associations were present in both groups of patients.

First, we examined associations of biomarker concentrations with the extent of atherosclerosis according to IVUS. We applied linear regression analyses with biomarker concentrations as the independent variable (ln-transformed or categorized when appropriate) and segmental plaque burden in the imaged coronary segment as the dependent variable. The results are presented as β's (per unit increase in ln-transformed biomarker concentration or per category of biomarker concentration) with 95% confidence intervals (95% CI). Subsequently, we examined the associations between biomarker concentrations and composition of atherosclerosis, specifically the presence of VH-TCFA lesions as well as VH-TCFA lesions with plaque burden ≥70%. We used logistic regression analyses with biomarker concentrations as the independent variable (ln-transformed or categorized when appropriate). The results are presented as odds ratios (ORs) per unit increase in ln-transformed biomarker concentration or per category of biomarker concentration, with 95% CI's.

First, all above-described analyses were performed univariably. Subsequently, we adjusted for age, gender, indication for coronary angiography, diabetes, hypertension and CRP. All data were analyzed with SPSS software (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY, USA). All statistical tests were two-tailed and p-values <0.05 were considered statistically significant.

Results

Baseline characteristics

Baseline characteristics are summarized in Table 1. Mean age was 61.5±11.4 years and 75.4% were men. Coronary angiography or PCI was performed for several indications: 159 (27.9%) patients had an acute myocardial infarction, 150 (26.3%) patients had unstable angina pectoris and 261 (45.8%) had SAP. The median length of the imaged coronary segment was 44.1 [33.7 - 55.4] mm. Based on IVUS-VH, a total of 239 (41.9%) patients had at least 1 TCFA lesion, including 69 (12.1%) patients with at least 1 TCFA lesion with a plaque burden ≥70%. Concentrations of IFN-γ, TNF R2, IL-8, IL-10 and IL-18 were not normally distributed; these biomarkers were therefore ln-transformed for further analyses. TNF-α, TNF-β and IL-6 were too low to detect in a large part of the patients, and thus were not examined as continuous variables in the statistical models. TNF-α was too low to detect in 24%, and hence was categorized into tertiles for further analyses. TNF-β and IL-6 were too low to detect in 92% and 62% of the patients, respectively, and these markers were dichotomized into measurable versus not measurable for further analyses. IL-10 concentrations could be measured in 99%. TNF R2, IL-8, IL-18 and IFN-γ were measurable in all patients.

Table 1. Baseline characteristics.

	Total (n = 570)	ACS patients (n = 309)	SAP patients (n = 261)
Patient characteristics			
Age, years	61.5 ± 11.4	59.7 ± 11.9	63.6 ± 10.3
Male gender, n (%)	430 (75.4)	227 (73.5)	203 (77.8)
Hypertension, n (%)	295 (51.8)	134 (43.4)	161 (61.7)
Hypercholesterolemia, n (%)	317 (55.6)	137 (44.3)	180 (69.0)
Diabetes mellitus, n (%)	99 (17.4)	40 (12.9)	59 (22.6)
Positive family history, n (%)	293 (51.4)	140 (45.3)	153 (58.6)
Smoking, n (%)	164 (28.8)	115 (37.2)	49 (18.8)
Peripheral artery disease, n (%)	36 (6.3)	12 (3.9)	24 (9.2)
Previous myocardial infarction, n (%)	184 (32.3)	80 (25.9)	104 (39.8)
Previous PCI, n (%)	185 (32.5)	57 (18.4)	128 (49.0)
Previous CABG, n (%)	18 (3.2)	7 (2.3)	11 (4.2)
Previous stroke, n (%)	23 (4.0)	10 (3.2)	13 (5.0)
History of renal insufficiency, n (%)	32 (5.6)	13 (4.2)	19 (7.3)
History of heart failure, n (%)	19 (3.3)	6 (1.9)	13 (5.0)

Table 1 continued.

	Total (n = 570)	ACS patients (n = 309)	SAP patients (n = 261)
Procedural characteristics			
PCI performed, n (%)	501 (87.9)	287 (92.9)	214 (82.0)
Indication for coronary angiography			
Acute coronary syndrome, n (%)	309 (54.2)	309 (100)	0 (0)
Myocardial infarction, n (%)	159 (27.9)	159 (51.5)	0 (0)
Unstable angina pectoris, n (%)	150 (26.3)	150 (48.5)	0 (0)
Stable angina pectoris, n (%)	261 (45.8)	0 (0)	261 (100)
Coronary artery disease			
No significant stenosis, n (%)	42 (7.4)	18 (5.8)	24 (9.2)
1-vessel disease, n (%)	301 (52.8)	168 (54.4)	133 (51.0)
2-vessel disease, n (%)	166 (29.1)	88 (28.5)	78 (29.9)
3-vessel disease, n (%)	61 (10.7)	35 (11.3)	26 (10.0)
IVUS segment characteristics			
Segment length, mm	44.1 [33.7-55.4]	43.9 [32.9-54.1]	44.8 [34.2-57.2]
Plaque burden, %	39.2 [30.0-46.4]	37.2 [28.0-45.5]	40.2 [31.8-47.8]
Presence of VH-TCFA, n (%)	239 (41.9)	140 (45.3)	99 (37.9)
Presence of VH-TCFA with PB ≥70%, n (%)	69 (12.1)	32 (10.4)	37 (14.2)
Serum biomarker concentrations			
C-reactive protein, mg/L	2.1 [0.8-5.3]	2.8 [1.1-7.0]	1.5 [0.6-3.1]
Tumor necrosis factor-α, pg/mL[a]	2.0 [1.4-2.9]	1.8 [1.4-2.6]	2.0 [1.4-3.3]
Tumor necrosis factor-β, pg/mL[b,c]	35.0 [18.0-116.0]	20.5 [16.5-44.3]	36.5 [27.0-152.8]
Tumor necrosis factor receptor 2, ng/mL[d]	4.5 [3.6-5.7]	4.4 [3.5-5.8]	4.5 [3.7-5.6]
Interferon-γ, pg/mL[c,e]	5.1 [3.9-7.3]	4.8 [3.8-6.6]	5.7 [4.2-8.2]
Interleukin-6, pg/mL[f]	3.5 [2.2-5.8]	3.7 [2.5-6.8]	2.5 [2.1-4.1]
Interleukin-8, pg/mL[c,d]	8.9 [6.8-12.0]	9.9 [7.1-12.6]	8.3 [6.5-10.3]
Interleukin-10, pg/mL[c,d]	5.2 [3.6-9.4]	6.9 [4.1-15.0]	4.4 [3.0-6.0]
Interleukin-18, pg/mL[e]	171.0 [132.3-215.0]	173.0 [133.0-216.3]	169.5 [130.5-211.3]

ACS=acute coronary syndrome; CABG=coronary artery bypass grafting; IVUS=intravascular ultrasound; PB=plaque burden; PCI=percutaneous coronary intervention; RANTES, Regulated upon Activation Normal T cell Expressed and Secreted; SAP=stable angina pectoris; VH-TCFA=virtual-histology thin-cap fibroatheroma. [a]Measurable in 76% of patients, too low to detect in 24%. [b]Measurable in 8% of patients, too low to detect in 92%. [c]TNFβ, IFN-γ, IL-10 and IL 18: total n=473, ACS n=309, SAP n=261. [d]Measurable in >99% of patients, too low to detect in <1%. [e]Measurable in all patients. [f]Measurable in 38% of patients, too low to detect in 62%.

Biomarkers and extent of atherosclerosis

The results of the analyses for plaque burden of the entire measured segment are shown in Figure 1 and Supplementary Table 1A, 1B and 1C. Higher TNF-α was associated with higher coronary plaque burden in patients with SAP (β (95% CI): 4.45 (0.99 - 7.91), for the highest vs the lowest tertile of TNF-α). Such an effect could not be demonstrated in patients with ACS.

Furthermore, lower IL-10 concentrations were associated with higher coronary plaque burden in the full cohort (β (95% CI): -3.88 (-6.00 - -1.76), for the highest vs the lowest tertile

of IL-10). This effect was driven by both the SAP patients and the ACS patients. Although effect estimates for the highest tertile of IL-10 were similar in both groups SAP: -2.95 (-6.23 - 0.33), ACS: -3.42 (-6.57 - -0.27), in the SAP patients the estimates, as well as the linear trend, did not reach statistical significance.

After multivariable adjustment, associations remained essentially the same for both TNF-α and IL-10.

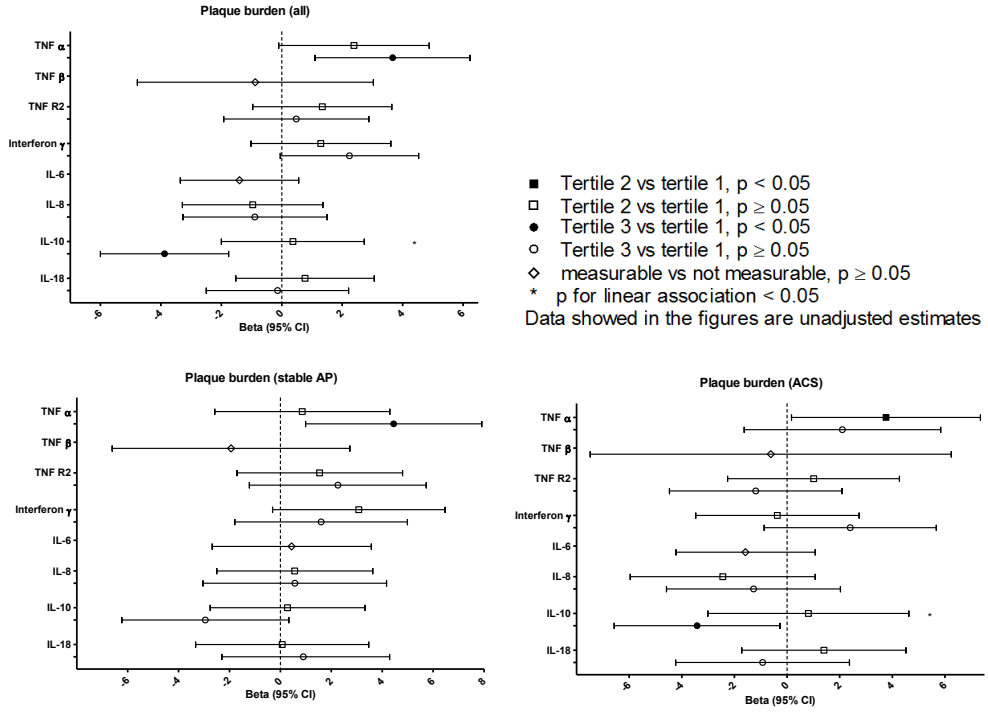


Figure 1. Association of TNF-α, TNF-β, TNF R2, IFN-γ, IL-6, IL-8, IL-10 and IL-18 with segment plaque burden in all patients, patients with stable AP and patients with ACS.

Biomarkers and composition of atherosclerosis

The results of the analyses for VH-TCFA lesions are displayed in Figure 2 and Supplementary Table 2A, 2B and 2C. High TNF-α was positively associated with presence of VH-TCFA lesions in patients with SAP (OR (95% CI): 2.30 (1.17 - 4.52) for the highest vs the lowest tertile of TNF-α). Such an effect was absent in patients with ACS. Furthermore, higher IL-8 seemed to confer lower risk of VH-TCFA in ACS patients; however, this effect was mainly driven by

tertile 2. No associations were present between any of the other biomarkers and VH-TCFA.

Higher TNF-α was positively associated with presence of VH-TCFA lesions with a plaque burden ≥70% in the full cohort (OR (95% CI): 2.85 (1.28 - 6.31) for the highest vs the lowest tertile of TNF-α) (Figure 3). This effect was driven by both patients with SAP and patients with ACS. Although the effect estimate reached statistical significance in the full cohort, this was not the case in the SAP and ACS groups. Nevertheless, the effect estimates for the highest tertile of TNF-α were similar in magnitude in both groups (SAP: 3.44 (0.89 - 13.29), ACS: 2.39 (0.89 - 6.45)). Higher IL-10 displayed an inverse association with presence of VH-TCFA lesions with a plaque burden ≥70% in the full cohort (OR (95% CI): 0.31 (0.12 - 0.80) for the highest vs the lowest tertile of IL-10, p for trend=0.037). Again, effect estimates did not reach statistical significance in these separate groups.

After multivariable adjustment, associations remained essentially the same.

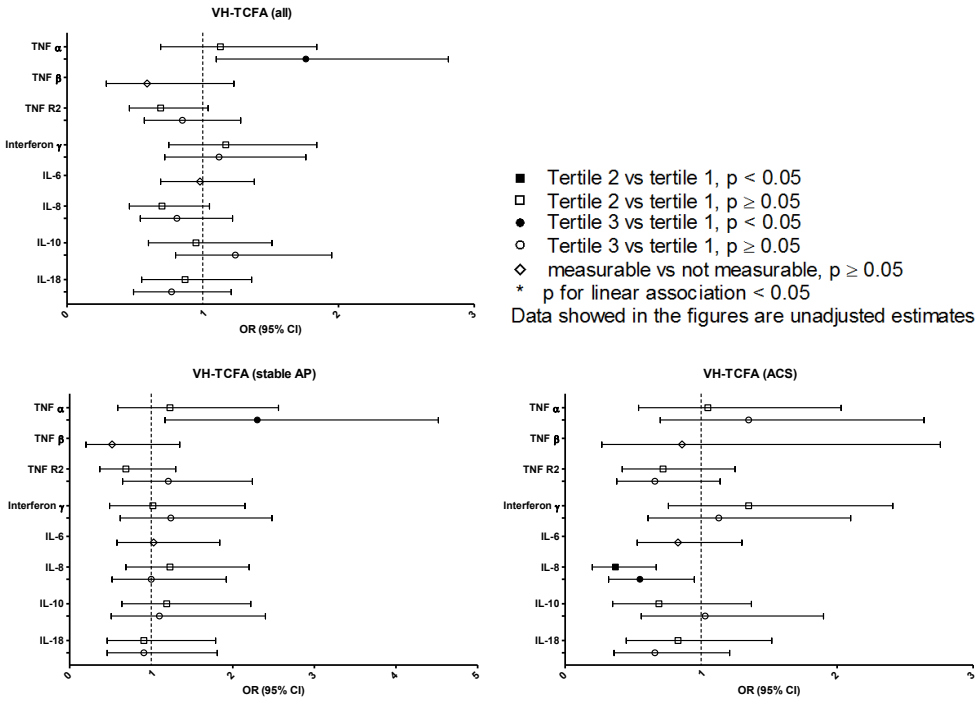


Figure 2. Association of TNF-α, TNF-β, TNF R2, IFN-γ, IL-6, IL-8, IL-10 and IL-18 with presence of VH-TCFA in all patients, patients with stable AP and patients with ACS.

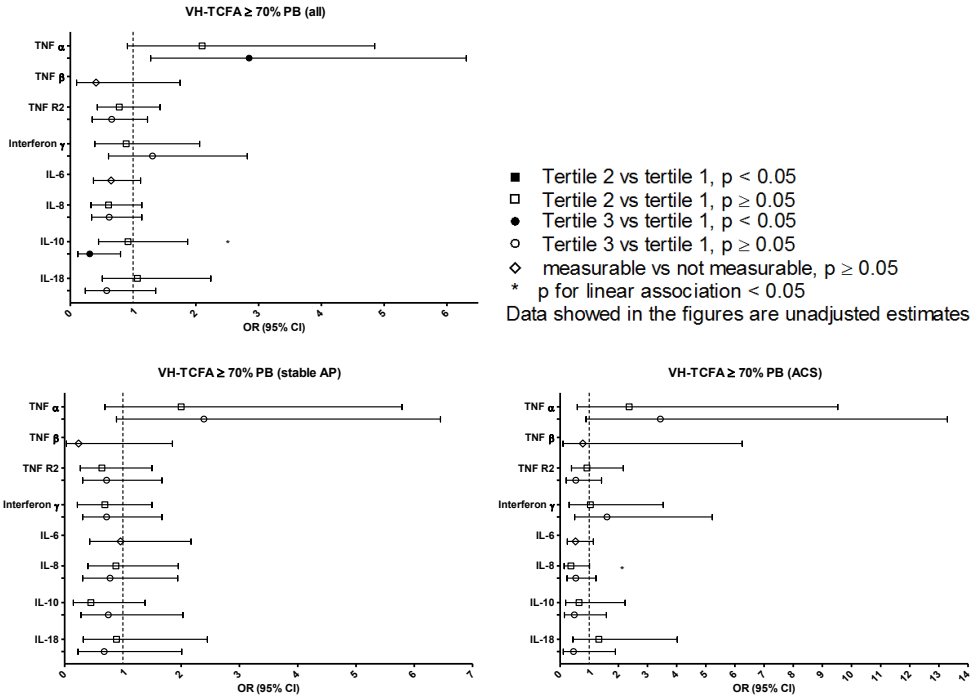


Figure 3. Association of TNF-a, TNF-b, TNF R2, INFg, IL-6, IL-8, IL-10 and IL-18 with presence of VH-TCFA with plaque burden ≥70% in all patients, patients with stable AP and patients with ACS.

Discussion

This study examined whether circulating cytokine concentrations are associated with extent and composition of coronary atherosclerosis, as determined by IVUS and IVUS-VH in a non-culprit vessel, in patients with SAP or ACS undergoing coronary angiography. In patients with SAP, higher concentrations of TNF-α were associated with higher coronary plaque burden and with presence of VH-TCFA lesions, and displayed a tendency towards a positive association with presence of VH-TCFA lesion with a plaque burden ≥70%. Overall, higher concentrations of IL-10 were inversely associated with coronary plaque burden and with presence of VH-TCFA with a plaque burden ≥70%. These effects of IL-10 did not reach statistical significance in the separate groups.

Inflammation is known to play a major role in atherosclerosis. In a previous study in the current patient population, we have demonstrated an association between CRP and IVUS characteristics [12]. TNF-α is a proinflammatory cytokine that is secreted from activated innate

immunity cells and is capable of inducing a cascade with a broad range of effects, including immunological activation, apoptosis, and procoagulative and antifibrinolytic actions, all of which can have an effect on the course of atherosclerosis [5,13]. Experimental studies on the role of TNF-a in plaque development and stability in mice have rendered inconsistent results, some finding anti-atherogenic effects and others finding pro-atherogenic effects [5]. This discrepancy in results may be due to differences in underlying mechanisms of atherogenesis in different types of mouse models. A recent study [14] in human saphenous vein organ culture, to which a combination of TNF-a and LDL was applied, demonstrated phenotypic changes characteristic of the initial development of atherosclerotic plaques. Clinical studies on the role of TNF-α in cardiovascular disease have also rendered inconsistent results. A prior study found an increase of serum TNF-α in patients with MI and unstable angina pectoris compared to healthy subjects [15]. Ridker et al. [16] found that plasma concentrations of TNF-α are persistently elevated among post-MI patients at increased risk for recurrent coronary events [17]. Furthermore, Naranjo et al. [18] found that TNF-α therapy was associated with a lower incidence of cardiovascular events in patients with rheumatoid arthritis, who are known to be at high cardiovascular risk. On the other hand, Cherneva et al. [19] and Sukhija et al. [20] examined the prognostic abilities of TNF-α in patients with known coronary artery disease, but did not find any associations between TNF-α and patient outcome. In the current study, we found that higher TNF-α level are associated with both extent of atherosclerosis and with plaque vulnerability in patients with SAP, which is in line with the presumed proinflammatory nature of this cytokine.

IL-10 is an anti-inflammatory cytokine that is produced by macrophages and lymphocytes [6]. This cytokine is capable of inhibiting many cellular processes that may play an important role in atherosclerotic lesion development and in the modulation of plaque composition [6,21]. Mallat et al. [21] investigated atherosclerotic lesions in IL-10 deficient mice and showed increased infiltration of inflammatory cells, increased production of IFN-γ, and decreased collagen content, which resulted in development of atheromatous lesions with signs of increased vulnerability. Several clinical studies have been performed on IL-10 and cardiovascular disease. Heeschen et al. [22] demonstrated that a reduced serum IL-10 level in patients with ACS is indicative of a poor prognosis. Most subsequent studies on the association of elevated circulating IL-10 levels with cardiovascular outcome have demonstrated positive associations with better prognosis [23-27]. In line with this, we found an inverse association between IL-10 and coronary plaque burden as well as between IL-10 and

presence of large, vulnerable plaques (i.e., VH-TCFA lesions with a plaque burden ≥70%) in the overall study population. However, we did not find an association of IL-10 with presence of TCFA lesions in general. These results suggest that IL-10 may in particular be associated with lower extent of coronary atherosclerosis and slower growth of VH-TCFAs. In any case, these findings further support the hypothesis of a protective role of IL-10 in atherosclerosis.

The associations of TNF-α and IL-10 with extent and composition of atherosclerosis demonstrated in the current study, suggest that these cytokines may potentially be useful for clinical risk stratification with regard to cardiovascular outcome. However, we did not find any associations between the cytokines we investigated and major adverse cardiac events during 1-year follow-up, adjusting for clinical covariates (Supplementary Table 4A-C). Possible explanations may include the fact that the magnitude of the effects of TNF-α and IL-10 may be relatively small in the context of this multifactorial disease, or that the current study lacks statistical power to expose these effects.

We did not find any associations between several cytokines we examined and the extent or composition of atherosclerosis. Analysis of some of the biomarkers (TNF-β and IL-6) was complicated by the fact that over 50% of the measurements were too low to detect. Cytokine assays are generally known to display limitations in terms of % detectability [28,29]. This makes clinical investigations into the pathophysiological role and the prognostic value of these biomarkers challenging. In line with this, few clinical studies have been performed on circulating TNF-β. Furthermore, IL-6 is known to have large circadian variations, and a relatively short half-life of less than 6h [30], which also makes this marker difficult to investigate. Clinical studies on circulating TNF R2, IFN-γ, and IL-8 in patients with coronary artery disease are also limited in number. IL-18 has been examined more often, and has been suggested to be associated with the presence and severity of coronary atherosclerosis [31,32]. In the present study, we could not demonstrate such an association.

Some aspects of this study warrant consideration. Our study population consisted of patients with SAP as well as patients with ACS. The group of patients with ACS is likely to be more heterogeneous, which may have influenced the findings. To account for this, we have performed the analyses separately in both groups. Furthermore, VH-IVUS imaging took place of a prespecified single target segment of a single non-culprit coronary artery, based on the assumption that such a non-stenotic segment adequately reflects coronary wall pathophysiology of the larger coronary tree. Although this assumption may be debated, previous studies evaluating IVUS have demonstrated that the coronary wall of comparable

non-culprit, non-stenotic segments of a single vessel does reflect coronary disease burden at large and is associated with subsequent cardiovascular outcome [33-35]. Moreover, it is important to note that IVUS is formally not capable of detecting the most rupture prone of all plaque phenotypes, the TCFA [36,37], because the spatial resolution of IVUS is insufficient for thin cap detection [23, 24]. Nonetheless, a concept of VH-IVUS derived TCFA has been postulated for plaques with a plaque burden >40% and a confluent necrotic core ≥10% in direct contact with the lumen in at least three VH-IVUS frames [13, 23]. Notably, we have recently demonstrated that such VH-IVUS derived TCFA lesions are strongly and independently predictive of the occurrence of major adverse cardiac events within the current study population [33].

In conclusion, in patients undergoing coronary angiography, higher circulating TNF-α was associated with higher plaque burden and with presence of VH-TCFA lesions in patients with SAP. Overall, lower circulating IL-10 was associated with higher plaque burden and with presence of VH-TCFA lesions with a plaque burden ≥70%. The latter effects did not reach statistical significance in the separate SAP and ACS groups. These in-vivo findings illustrate that TNF-α and IL-10 appear to play a role in both extent and vulnerability of coronary atherosclerosis, which is in line with experimental studies.

References

1. Libby P. Inflammation in atherosclerosis. Arterioscler Thromb Vasc Biol 2012 Sep; 32: 2045-2051.
2. Hansson G.K. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 2005; 352: 1685-1695.
3. Ross R. Atherosclerosisean inflammatory disease. N Engl J Med 1999; 340: 115-126.
4. Libby P. Mechanisms of acute coronary syndromes and their implications for therapy. N Engl J Med 2013; 368: 2004-2013.
5. Tedgui A. and Mallat Z. Cytokines in atherosclerosis: pathogenic and regulatory pathways. Physiol Rev 2006; 86: 515-581.
6. Ait-Oufella H. et al. Recent advances on the role of cytokines in atherosclerosis. Arterioscler Thromb Vasc Biol 2011; 31: 969-979.
7. Hansson G.K. et al. Inflammation and atherosclerosis. Annu Rev Pathol 2006; 1: 297-329.
8. Voloshyna I. et al. Atherosclerosis and interferon-gamma: New insights and therapeutic targets. Trends Cardiovasc Med 2014; 24: 45-51.
9. de Boer S.P. et al. Relation of genetic profile and novel circulating biomarkers with coronary plaque phenotype as determined by intravascular ultrasound: rationale and design of the ATHEROREMO-IVUS study. EuroIntervention: journal of EuroPCR in collaboration with the Working Group on Interventional Cardiology of the European Society of Cardiology 2013; pii: 20130113-01.
10. Nair A. et al. Automated coronary plaque characterisation with intravascular ultrasound backscatter: ex vivo validation. EuroIntervention: journal of EuroPCR in collaboration with the Working Group on Interventional Cardiology of the European Society of Cardiology 2007; 3: 113-120.
11. Rodriguez-Granillo G.A. et al. In vivo intravascular ultrasound-derived thin-cap fibroatheroma detection using ultrasound radiofrequency data analysis. J Am Coll Cardiol 2005; 46: 2038-2042.
12. Cheng J.M. et al. C-reactive protein in relation to coronary plaque burden and presence of high risk lesions on intravascular ultrasound and cardiovascular outcome: Results of the ATHEROREMO-IVUS study. Am J Cardiol 2014; 114: 1497-1503. doi: 10.1016/j.amjcard.2014.08.013.
13. Yudkin J.S. et al. C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial dysfunction: a potential role for cytokines originating from adipose tissue? Arterioscler Thromb Vasc Biol 1999; 19: 972-978.
14. Prasongsukarn K. et al. Phenotypic alterations in human saphenous vein culture induced by tumor necrosis factor-alpha and lipoproteins: a preliminary development of an initial atherosclerotic plaque model. Lipids Health Dis 2013; 12: 132.
15. Mizia-Stec K. et al. Serum tumour necrosis factor-alpha, interleukin-2 and interleukin-10 activation in stable angina and acute coronary syndromes. Coron Artery Dis 2003; 14: 431-438.
16. Ridker P.M. et al. Elevation of tumor necrosis factor-alpha and increased risk of recurrent coronary events after myocardial infarction. Circulation 2000; 101: 2149-2153.
17. Valgimigli M. et al. Tumor necrosis factor-alpha receptor 1 is a major predictor of mortality and new-onset heart failure in patients with acute myocardial infarction: the cytokine-activation and long-term prognosis in myocardial infarction (CALPHA) study. Circulation 2005; 111: 863-870.
18. Naranjo A. et al. Cardiovascular disease in patients with rheumatoid arthritis: results from the QUEST-RA study. Arthritis research & therapy 2008; 10: R30.
19. Cherneva Z.V. et al. Inflammatory cytokines at admissioneindependent prognostic markers in patients with

acute coronary syndrome and hyperglycaemia. Acute Card Care 2012; 14: 13-19.
20. Sukhija R. et al. Inflammatory markers, angiographic severity of coronary artery disease, and patient outcome. Am J Cardiol 2007; 99: 879-884.
21. Mallat Z. et al. Protective role of interleukin-10 in atherosclerosis. Circulation Res 1999; 85: e17-24.
22. Heeschen C. et al. Serum level of the antiinflammatory cytokine interleukin-10 is an important prognostic determinant in patients with acute coronary syndromes. Circulation 2003; 107: 2109-2114.
23. Welsh P. et al. Circulating interleukin-10 and risk of cardiovascular events: a prospective study in the elderly at risk. Arteriosclerosis. Thromb Vasc Biol 2011; 31: 2338-2344.
24. Oemrawsingh R.M. et al. Multimarker risk model containing troponin-T, interleukin 10, myeloperoxidase and placental growth factor predicts long-term cardiovascular risk after non-ST-segment elevation acute coronary syndrome. Heart 2011; 97: 1061-1066.
25. Chang L.T. et al. Role of stromal cell derived factor-1alpha, level and value of circulating interleukin-10 and endothelial progenitor cells in patients with acute myocardial infarction undergoing primary coronary angioplasty. Circ J 2009; 73: 1097-1104.
26. Yip H.K. et al. Association of interleukin-10 level with increased 30-day mortality in patients with ST-segment elevation acute myocardial infarction undergoing primary coronary intervention. Circ J 2007; 71: 1086-1091.
27. Anguera I. et al. Elevation of serum levels of the anti-inflammatory cytokine interleukin-10 and decreased risk of coronary events in patients with unstable angina. Am Heart J 2002; 144: 811-817.
28. Chaturvedi A.K. et al. Evaluation of multiplexed cytokine and inflammation marker measurements: a methodologic study. Cancer Epidemiol Biomarkers Prev 2011; 20: 1902-1911.
29. Soares H.D. et al. Identifying early markers of Alzheimer's disease using quantitative multiplex proteomicimmunoassay panels. Ann N Y Acad Sci 2009; 1180: 56-67.
30. Ridker P.M. et al. Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. Circulation 2000; 101: 1767-1772.
31. Rosso R. et al. Serum levels of interleukin-18 in patients with stable and unstable angina pectoris. Int J Cardiol 2005; 98: 45-48.
32. Hulthe J. et al. Plasma interleukin (IL)-18 concentrations is elevated in patients with previous myocardial infarction and related to severity of coronary atherosclerosis independently of C-reactive protein and IL-6. Atherosclerosis 2006; 188: 450-454.
33. Cheng J.M. et al. In vivo detection of high-risk coronary plaques by radiofrequency intravascular ultrasound and cardiovascular outcome: results of the ATHEROREMO-IVUS study. Eur Heart J 2014; 35: 639-647.
34. Nicholls S.J. et al. Intravascular ultrasound-derived measures of coronary atherosclerotic plaque burden and clinical outcome. J Am Coll Cardiol 2010; 55: 2399-2407.
35. Puri R. et al. Coronary atheroma volume and cardiovascular events during maximally intensive statin therapy. Eur Heart J 2013; 34: 3182-3190.
36. Garcia-Garcia H.M. et al. Imaging of coronary atherosclerosis: intravascular ultrasound. Eur Heart J 2010; 31: 2456-2469.
37. Virmani R. Are our tools for the identification of TCFA ready and do we know them? JACC Cardiovasc imaging 2011; 4: 656-658.

Supplementary files

Supplementary Table 1A. Association of TNF-α, TNF-β, TNF R2, IFN-γ, IL-6, IL-8, IL-10 and IL-18 with segment plaque burden in all patients.

Segment plaque burden	Unadjusted model		Multivariable model[a]	
	beta (95% CI)	P	beta (95% CI)	P
TNF-α				
Tertile 1	reference		reference	
Tertile 2	2.39 (-0.10-4.88)	0.060	1.94 (-0.52-4.39)	0.12
Tertile 3	3.67 (1.10-6.23)	0.005	3.13 (0.63-5.62)	0.014
TNF-β				
Not measurable	reference		reference	
Measurable	-0.88 (-4.78-3.03)	0.66	-1.39 (-5.25- -2.47)	0.48
TNF R2				
Tertile 1	reference		reference	
Tertile 2	1.34 (-0.96-3.64)	0.25	-0.56 (-2.89-1.76)	0.63
Tertile 3	0.48 (-1.92-2.88)	0.69	-1.73 (-4.29-0.82)	0.18
Ln (TNF R2)	0.61 (-1.98-3.20)	0.65	-2.43 (-5.15-0.29)	0.080
Interferon-γ				
Tertile 1	reference		reference	
Tertile 2	1.29 (-1.02 -3.61)	0.27	0.41 (-1.86-2.67)	0.73
Tertile 3	2.24 (-0.05-4.53)	0.055	0.51 (-1.98-2.99)	0.69
Ln (Interferon-γ)	1.61 (-0.15-3.37)	0.072	0.11 (-1.71-1.92)	0.91
IL-6				
Not measurable	reference		reference	
Measurable	-1.40 (-3.36-0.56)	0.16	-0.70 (-2.74- 1.35)	0.50
IL-8				
Tertile 1	reference		reference	
Tertile 2	-0.96 (-3.29-1.36)	0.42	-0.78 (-3.06-1.49)	0.50
Tertile 3	-0.89 (-3.27-1.50)	0.46	-1.63 (-4.08-0.82)	0.19
Ln (IL-8)	-0.07 (-2.22-2.09)	0.95	-0.54 (-2.70-1.62)	0.63
IL-10				
Tertile 1	reference		reference	
Tertile 2	0.37 (-2.00-2.73)	0.76	0.63 (-1.73-3.00)	0.60
Tertile 3	-3.88 (-6.00- -1.76)	<0.001	-3.27 (-5.55- -0.99)	0.005
Ln (IL-10)	-1.52 (-2.49- -0.55)	0.002	-1.25 (-2.26- -0.24)	0.016
IL-18				
Tertile 1	reference		reference	
Tertile 2	0.77 (-1.52-3.06)	0.51	1.04 (-1.24-3.33)	0.37
Tertile 3	-0.14 (-2.50-2.21)	0.91	0.14 (-2.15-2.42)	0.91
Ln (IL-18)	-0.84 (-3.17-1.48)	0.48	-0.53 (-2.80-1.74)	0.65

[a]Adjusted for age, gender, indication for coronary angiography, diabetes, hypertension, and CRP.

Supplementary Table 1B. Association of TNF-α, TNF-β, TNF R2, IFN-γ, IL-6, IL-8, IL-10 and IL-18 with segment plaque burden in patients with stable AP.

Segment plaque burden	Unadjusted model		Multivariable model[a]	
	beta (95% CI)	P	beta (95% CI)	P
TNF-α				
Tertile 1	reference		reference	
Tertile 2	0.86 (-2.58-4.30)	0.62	0.33 (-3.09-3.74)	0.85
Tertile 3	4.45 (0.99-7.91)	0.012	4.64 (1.11-8.16)	0.010
TNF-β				
Not measurable	reference		reference	
Measurable	-1.94 (-6.61-2.73)	0.41	-1.63 (-6.27-3.00)	0.49
TNF R2				
Tertile 1	reference		reference	
Tertile 2	1.54 (-1.71-4.80)	0.35	-0.16 (-3.49-3.18)	0.93
Tertile 3	2.26 (-1.22-5.73)	0.20	0.40 (-3.48-4.29)	0.84
Ln (TNF R2)	2.90 (-0.94-6.74)	0.14	0.64 (-3.54-4.82)	0.76
Interferon-γ				
Tertile 1	reference		reference	
Tertile 2	3.08 (-0.31-6.47)	0.075	2.57 (-0.92-6.05)	0.15
Tertile 3	1.60 (-1.79-4.99)	0.35	0.40 (-3.22-4.02)	0.83
Ln (Interferon-γ)	1.39 (-1.01-3.80)	0.26	0.44 (-2.07-2.95)	0.73
IL-6				
Not measurable	reference		reference	
Measurable	0.44 (-2.68-3.57)	0.78	0.47 (-2.76-3.70)	0.78
IL-8				
Tertile 1	reference		reference	
Tertile 2	0.56 (-2.50-3.63)	0.72	0.17 (-2.90-3.23)	0.91
Tertile 3	0.57 (-3.04-4.17)	0.76	-0.18 (-3.87-3.50)	0.92
Ln (IL-8)	2.03 (-1.11-5.16)	0.21	1.10 (-2.08-4.28)	0.50
IL-10				
Tertile 1	reference		reference	
Tertile 2	0.28 (-2.76-3.32)	0.86	0.34 (-2.65-3.33)	0.82
Tertile 3	-2.95 (-6.23-0.33)	0.078	-3.30 (-6.64-0.04)	0.053
Ln (IL-10)	-1.03 (-3.02-0.95)	0.31	-1.34 (-3.34-0.66)	0.19
IL-18				
Tertile 1	reference		reference	
Tertile 2	0.07 (-3.33-3.47)	0.97	-0.34 (-3.71-3.02)	0.84
Tertile 3	0.99 (-2.30-4.29)	0.55	0.11 (-3.24-3.47)	0.95
Ln (IL-18)	1.72 (-1.83-5.28)	0.34	0.99 (-2.57-4.56)	0.58

[a]Adjusted for age, gender, indication for coronary angiography, diabetes, hypertension, and CRP.

Supplementary Table 1C. Association of TNF-α, TNF-β, TNF R2, IFN-γ, IL-6, IL-8, IL-10 and IL-18 with segment plaque burden in patients with ACS.

Segment plaque burden	Unadjusted model		Multivariable model[a]	
	beta (95% CI)	P	beta (95% CI)	P
TNF-α				
Tertile 1	reference		reference	
Tertile 2	3.76 (0.17-7.35)	0.040	2.98 (-0.65-6.61)	0.11
Tertile 3	2.10 (-1.63-5.84)	0.27	1.79 (-1.83-5.41)	0.33
TNF-β				
Not measurable	reference		reference	
Measurable	-0.62 (-7.48-6.24)	0.86	-1.08 (-7.98-5.82)	0.76
TNF R2				
Tertile 1	reference		reference	
Tertile 2	1.01 (-2.26-4.27)	0.54	-1.19 (-4.48-2.10)	0.48
Tertile 3	-1.19 (-4.47-2.09)	0.48	-3.37 (-6.86-0.13)	0.059
Ln (TNF R2)	-1.18 (-4.67-2.30)	0.51	-4.53 (-8.17- -0.89)	0.015
Interferon-γ				
Tertile 1	reference		reference	
Tertile 2	-0.37 (-3.47-2.74)	0.82	-0.96 (-4.01-2.08)	0.53
Tertile 3	2.40 (-0.87-5.67)	0.15	0.58 (-2.89-4.05)	0.74
Ln (Interferon-γ)	1.09 (-1.51-3.70)	0.41	-0.22 (-2.89-2.46)	0.87
IL-6				
Not measurable	reference		reference	
Measurable	-1.58 (-4.22-1.07)	0.24	-1.49 (-4.18-1.20)	0.28
IL-8				
Tertile 1	reference		reference	
Tertile 2	-2.44 (-5.96-1.07)	0.17	-2.25 (-5.71-1.22)	0.20
Tertile 3	-1.27 (-4.58-2.03)	0.45	-2.77 (-6.12-0.59)	0.11
Ln (IL-8)	-0.99 (-3.99-2.02)	0.52	-2.02 (-5.02-0.97)	0.19
IL-10				
Tertile 1	reference		reference	
Tertile 2	0.81 (-3.01-4.63)	0.68	1.31 (-2.65-5.28)	0.51
Tertile 3	-3.42 (-6.57- -0.27)	0.034	-3.12 (-6.24-0.01)	0.051
Ln (IL-10)	-1.30 (-2.52- -0.08)	0.038	-1.27 (-2.48- -0.05)	0.041
IL-18				
Tertile 1	reference		reference	
Tertile 2	1.40 (-1.72-4.52)	0.38	2.11 (-1.14-5.35)	0.20
Tertile 3	-0.93 (-4.23-2.37)	0.58	0.07 (-3.21-3.34)	0.97
Ln (IL-18)	-2.30 (-5.35-0.75)	0.14	-1.52 (-4.55-1.51)	0.32

[a]Adjusted for age, gender, indication for coronary angiography, diabetes, hypertension, and CRP.

Supplementary Table 2A. Association of TNF-α, TNF-β, TNF R2, IFN-γ, IL-6, IL-8, IL-10 and IL-18 with presence of VH-TCFA in all patients.

VH-TCFA	Unadjusted model		Multivariable model[a]	
	OR (95% CI)	P	OR (95% CI)	P
TNF-α				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	1.13 (0.69-1.84)	0.63	1.12 (0.68-1.83)	0.67
Tertile 3	1.76 (1.10-2.81)	0.018	1.82 (1.13-2.93)	0.014
TNF-β				
Not measurable	1 (reference)		1 (reference)	
Measurable	0.59 (0.29-1.23)	0.16	0.70 (0.33-1.47)	0.34
TNF R2				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	0.69 (0.46-1.04)	0.079	0.68 (0.44-1.04)	0.078
Tertile 3	0.85 (0.57-1.28)	0.45	0.84 (0.54-1.30)	0.43
Ln (TNF R2)	0.87 (0.55-1.37)	0.55	0.85 (0.52-1.40)	0.52
Interferon-γ				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	1.17 (0.75-1.84)	0.50	1.21 (0.76-1.91)	0.42
Tertile 3	1.12 (0.72-1.76)	0.62	1.22 (0.75-1.97)	0.43
Ln (Interferon-γ)	1.08 (0.76-1.52)	0.68	1.15 (0.79-1.66)	0.47
IL-6				
Not measurable	1 (reference)		1 (reference)	
Measurable	0.98 (0.69-1.38)	0.90	0.97 (0.67-1.41)	0.87
IL-8				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	0.70 (0.46-1.05)	0.085	0.69 (0.46-1.06)	0.089
Tertile 3	0.81 (0.54-1.22)	0.81	0.77 (0.50-1.18)	0.23
Ln (IL-8)	0.91 (0.62-1.33)	0.63	0.87 (0.59-1.30)	0.50
IL-10				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	0.95 (0.60-1.51)	0.84	0.95 (0.59-1.52)	0.83
Tertile 3	1.24 (0.80-1.95)	0.34	1.21 (0.75-1.94)	0.44
Ln (IL-10)	1.15 (0.95-1.39)	0.16	1.13 (0.92-1.39)	0.25
IL-18				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	0.87 (0.55-1.36)	0.54	0.90 (0.57-1.43)	0.66
Tertile 3	0.77 (0.49-1.21)	0.25	0.76 (0.48-1.20)	0.24
Ln (IL-18)	0.90 (0.57-1.42)	0.64	0.91 (0.57-1.44)	0.67

[a]Adjusted for age, gender, indication for coronary angiography, diabetes, hypertension, and CRP.

Supplementary Table 2B. Association of TNF-α, TNF-β, TNF R2, IFN-γ, IL-6, IL-8, IL-10 and IL-18 with presence of VH-TCFA in patients with stable AP.

VH-TCFA	Unadjusted model		Multivariable model[a]	
	OR (95% CI)	P	OR (95% CI)	P
TNF-α				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	1.23 (0.59-2.56)	0.58	1.27 (0.60-2.66)	0.53
Tertile 3	2.30 (1.17-4.52)	0.015	2.31 (1.16-4.59)	0.017
TNF-β				
Not measurable	1 (reference)		1 (reference)	
Measurable	0.52 (0.20-1.35)	0.18	0.52 (0.20-1.37)	0.19
TNF R2				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	0.69 (0.37-1.30)	0.25	0.67 (0.35-1.29)	0.23
Tertile 3	1.21 (0.65-2.24)	0.55	1.14 (0.58-2.23)	0.71
Ln (TNF R2)	1.44 (0.70-2.94)	0.32	1.38 (0.62-3.04)	0.43
Interferon-γ				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	1.02 (0.49-2.15)	0.95	0.96 (0.45-2.05)	0.91
Tertile 3	1.24 (0.62-2.48)	0.55	1.19 (0.57-2.50)	0.64
Ln (Interferon-γ)	1.23 (0.74-2.05)	0.43	1.23 (0.71-2.13)	0.45
IL-6				
Not measurable	1 (reference)		1 (reference)	
Measurable	1.03 (0.58-1.84)	0.92	0.95 (0.51-1.76)	0.87
IL-8				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	1.23 (0.69-2.20)	0.48	1.27 (0.70-2.29)	0.44
Tertile 3	1.00 (0.52-1.92)	1.00	0.95 (0.48-1.85)	0.87
Ln (IL-8)	1.15 (0.64-2.05)	0.64	1.08 (0.59-1.97)	0.81
IL-10				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	1.19 (0.64-2.22)	0.58	1.22 (0.65-2.30)	0.54
Tertile 3	1.10 (0.51-2.40)	0.81	1.06 (0.47-2.36)	0.90
Ln (IL-10)	1.41 (0.93-2.15)	0.11	1.39 (0.90-2.14)	0.14
IL-18				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	0.91 (0.46-1.79)	0.78	0.88 (0.44-1.76)	0.72
Tertile 3	0.91 (0.46-1.81)	0.78	0.82 (0.41-1.68)	0.60
Ln (IL-18)	1.01 (0.48-2.13)	0.99	0.95 (0.44-2.04)	0.89

[a]Adjusted for age, gender, indication for coronary angiography, diabetes, hypertension, and CRP.

Supplementary Table 2C. Association of TNF-α, TNF-β, TNF R2, IFN-γ, IL-6, IL-8, IL-10 and IL-18 with presence of VH-TCFA in patients with ACS.

VH-TCFA	Unadjusted model		Multivariable model[a]	
	OR (95% CI)	P	OR (95% CI)	P
TNF-α				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	1.05 (0.54-2.03)	0.89	0.89 (0.45-1.78)	0.74
Tertile 3	1.35 (0.70-2.64)	0.37	1.43 (0.72-2.84)	0.31
TNF-β				
Not measurable	1 (reference)		1 (reference)	
Measurable	0.86 (0.27-2.76)	0.80	0.98 (0.29-3.34)	0.98
TNF R2				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	0.72 (0.42-1.25)	0.25	0.69 (0.39-1.22)	0.20
Tertile 3	0.66 (0.38-1.14)	0.14	0.62 (0.34-1.12)	0.11
Ln (TNF R2)	0.63 (0.34-1.14)	0.13	0.59 (0.30-1.15)	0.12
Interferon-γ				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	1.35 (0.76-2.41)	0.30	1.38 (0.77-2.49)	0.28
Tertile 3	1.13 (0.61-2.10)	0.69	1.15 (0.60-2.21)	0.68
Ln (Interferon-γ)	1.06 (0.65-1.73)	0.83	1.05 (0.63-1.76)	0.86
IL-6				
Not measurable	1 (reference)		1 (reference)	
Measurable	0.83 (0.53-1.30)	0.42	0.96 (0.59-1.55)	0.86
IL-8				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	0.37 (0.20-0.67)	0.001	0.40 (0.21-0.74)	0.004
Tertile 3	0.55 (0.32-0.95)	0.033	0.60 (0.33-1.08)	0.086
Ln (IL-8)	0.70 (0.42-1.17)	0.17	0.76 (0.44-1.30)	0.31
IL-10				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	0.69 (0.35-1.37)	0.29	0.76 (0.37-1.54)	0.44
Tertile 3	1.03 (0.56-1.90)	0.93	1.14 (0.61-2.14)	0.68
Ln (IL-10)	1.02 (0.81-1.28)	0.90	1.03 (0.82-1.31)	0.79
IL-18				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	0.83 (0.45-1.52)	0.55	0.90 (0.48-1.69)	0.75
Tertile 3	0.66 (0.36-1.21)	0.18	0.65 (0.35-1.21)	0.17
Ln (IL-18)	0.82 (0.46-1.46)	0.50	0.82 (0.46-1.49)	0.52

[a]Adjusted for age, gender, indication for coronary angiography, diabetes, hypertension, and CRP.

Supplementary Table 3A. Association of TNF-α, TNF-β, TNF R2, IFN-γ, IL-6, IL-8, IL-10 and IL-18 with presence of VH-TCFA with plaque burden ≥70% in all patients.

VH-TCFA with PB ≥70%	Unadjusted model		Multivariable model[a]	
	OR (95% CI)	P	OR (95% CI)	P
TNF-α				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	2.10 (0.91-4.85)	0.083	2.11 (0.91-4.93)	0.084
Tertile 3	2.85 (1.28-6.31)	0.01	2.78 (1.24-6.23)	0.013
TNF-β				
Not measurable	1 (reference)		1 (reference)	
Measurable	0.41 (0.10-1.75)	0.23	0.41 (0.10-1.78)	0.24
TNF R2				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	0.78 (0.43-1.43)	0.42	0.67 (0.36-1.25)	0.20
Tertile 3	0.66 (0.35-1.23)	0.19	0.52 (0.26-1.04)	0.064
Ln (TNF R2)	0.65 (0.32-1.30)	0.22	0.50 (0.23-1.09)	0.081
Interferon-γ				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	0.89 (0.39-2.06)	0.79	0.78 (0.34-1.83)	0.57
Tertile 3	1.31 (0.61-2.82)	0.49	0.93 (0.41-2.14)	0.87
Ln (Interferon-γ)	1.21 (0.66-2.21)	0.54	0.93 (0.48-1.80)	0.83
IL-6				
Not measurable	1 (reference)		1 (reference)	
Measurable	0.65 (0.37-1.12)	0.12	0.75 (0.42-1.36)	0.35
IL-8				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	0.61 (0.33-1.14)	0.12	0.63 (0.34-1.18)	0.15
Tertile 3	0.62 (0.34-1.14)	0.12	0.64 (0.34-1.22)	0.17
Ln (IL-8)	0.57 (0.32-1.02)	0.059	0.57 (0.31-1.05)	0.069
IL-10				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	0.92 (0.45-1.87)	0.81	0.97 (0.47-2.02)	0.94
Tertile 3	0.31 (0.12-0.80)	0.016	0.36 (0.13-0.97)	0.043
Ln (IL-10)	0.64 (0.42-0.97)	0.037	0.69 (0.44-1.08)	0.10
IL-18				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	1.07 (0.51-2.24)	0.87	1.09 (0.51-2.32)	0.82
Tertile 3	0.58 (0.24-1.36)	0.21	0.59 (0.25-1.40)	0.23
Ln (IL-18)	0.49 (0.23-1.08)	0.077	0.51 (0.22-1.14)	0.10

[a]Adjusted for age, gender, indication for coronary angiography, diabetes, hypertension, and CRP.

Supplementary Table 3B. Association of TNF-α, TNF-β, TNF R2, IFN-γ, IL-6, IL-8, IL-10 and IL-18 with presence of VH-TCFA with plaque burden ≥70% in patients with stable AP.

VH-TCFA with PB ≥70%	Unadjusted model		Multivariable model[a]	
	OR (95% CI)	P	OR (95% CI)	P
TNF-α				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	2.00 (0.69-5.79)	0.20	2.11 (0.72-6.18)	0.17
Tertile 3	2.39 (0.89-6.45)	0.086	2.48 (0.90-6.79)	0.078
TNF-β				
Not measurable	1 (reference)		1 (reference)	
Measurable	0.24 (0.03-1.85)	0.17	0.24 (0.03-1.86)	0.17
TNF R2				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	0.64 (0.27-1.50)	0.30	0.63 (0.26-1.53)	0.31
Tertile 3	0.72 (0.31-1.67)	0.45	0.71 (0.28-1.77)	0.46
Ln (TNF R2)	0.79 (0.29-2.15)	0.65	0.81 (0.27-2.42)	0.70
Interferon-γ				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	0.69 (0.22-2.19)	0.53	0.64 (0.20-2.05)	0.45
Tertile 3	0.90 (0.32-2.53)	0.85	0.83 (0.28-2.47)	0.73
Ln (Interferon-γ)	0.96 (0.44-2.09)	0.91	0.90 (0.38-2.12)	0.81
IL-6				
Not measurable	1 (reference)		1 (reference)	
Measurable	0.96 (0.43-2.17)	0.93	0.99 (0.42-2.33)	0.99
IL-8				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	0.88 (0.40-1.95)	0.76	0.91 (0.41-2.04)	0.82
Tertile 3	0.78 (0.31-1.94)	0.59	0.79 (0.31-2.00)	0.62
Ln (IL-8)	0.85 (0.38-1.94)	0.70	0.87 (0.37-2.01)	0.74
IL-10				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	1.19 (0.50-2.88)	0.69	1.23 (0.50-2.98)	0.66
Tertile 3	0.75 (0.28-2.03)	0.57	0.74 (0.27-2.05)	0.57
Ln (IL-10)	0.66 (0.32-1.36)	0.26	0.64 (0.30-1.36)	0.25
IL-18				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	0.89 (0.32-2.45)	0.82	0.87 (0.31-2.42)	0.79
Tertile 3	0.68 (0.23-2.01)	0.48	0.63 (0.21-1.93)	0.42
Ln (IL-18)	0.55 (0.18-1.71)	0.30	0.53 (0.17-1.68)	0.28

[a]Adjusted for age, gender, indication for coronary angiography, diabetes, hypertension, and CRP.

Supplementary Table 3C. Association of TNF-α, TNF-β, TNF R2, IFN-γ, IL-6, IL-8, IL-10 and IL-18 with presence of VH-TCFA with plaque burden ≥70% in patients with ACS.

VH-TCFA with PB ≥70%	Unadjusted model		Multivariable model[a]	
	OR (95% CI)	P	OR (95% CI)	P
TNF-α				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	2.37 (0.59-9.53)	0.23	2.07 (0.50-8.65)	0.32
Tertile 3	3.44 (0.89-13.29)	0.073	3.57 (0.90-14.13)	0.070
TNF-β				
Not measurable	1 (reference)		1 (reference)	
Measurable	0.78 (0.10-6.25)	0.82	0.86 (0.10-7.15)	0.89
TNF R2				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	0.92 (0.39-2.17)	0.86	0.66 (0.27-1.66)	0.38
Tertile 3	0.54 (0.21-1.42)	0.22	0.35 (0.12-1.03)	0.056
Ln (TNF R2)	0.51 (0.19-1.39)	0.19	0.30 (0.09-0.97)	0.044
Interferon-γ				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	1.04 (0.31-3.54)	0.95	1.02 (0.29-3.56)	0.98
Tertile 3	1.61 (0.50-5.22)	0.43	1.12 (0.32-3.86)	0.86
Ln (Interferon-γ)	1.40 (0.53-3.71)	0.50	1.02 (0.37-2.84)	0.97
IL-6				
Not measurable	1 (reference)		1 (reference)	
Measurable	0.53 (0.25-1.14)	0.10	0.60 (0.27-1.36)	0.22
IL-8				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	0.37 (0.14-1.01)	0.052	0.38 (0.13-1.07)	0.066
Tertile 3	0.54 (0.24-1.23)	0.14	0.50 (0.20-1.23)	0.13
Ln (IL-8)	0.42 (0.18-0.96)	0.039	0.38 (0.16-0.91)	0.029
IL-10				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	0.65 (0.19-2.23)	0.49	0.69 (0.19-2.50)	0.57
Tertile 3	0.49 (0.15-1.59)	0.24	0.53 (0.16-1.79)	0.31
Ln (IL-10)	0.69 (0.40-1.20)	0.19	0.71 (0.41-1.23)	0.22
IL-18				
Tertile 1	1 (reference)		1 (reference)	
Tertile 2	1.33 (0.44-4.02)	0.61	1.37 (0.43-4.42)	0.60
Tertile 3	0.46 (0.11-1.90)	0.28	0.52 (0.12-2.21)	0.37
Ln (IL-18)	0.44 (0.14-1.35)	0.15	0.45 (0.13-1.54)	0.20

[a]Adjusted for age, gender, indication for coronary angiography, diabetes, hypertension, and CRP.

Supplementary Table 4A. Association of TNF-α, TNF-β, TNF R2, IFN-γ, IL-6, IL-8, IL-10 and IL-18 with occurrence of major adverse cardiac events in all patients.

MACE	Unadjusted model		Multivariable model[a]		Multivariable model[b]	
	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
TNF-α						
Tertile 1	1 (reference)		1 (reference)		1 (reference)	
Tertile 2	0.95 (0.48-1.90)	0.89	0.88 (0.44-1.77)	0.73	0.96 (0.48-1.93)	0.91
Tertile 3	0.82 (0.40-1.65)	0.57	0.76 (0.37-1.54)	0.44	0.74 (0.36-1.51)	0.40
TNF-β						
Not measurable	1 (reference)		1 (reference)		1 (reference)	
Measurable	0.53 (0.13-2.19)	0.38	0.51 (0.12-2.08)	0.34	0.54 (0.13-2.23)	0.40
TNF R2						
Tertile 1	1 (reference)		1 (reference)		1 (reference)	
Tertile 2	1.06 (0.51-2.19)	0.88	0.88 (0.42-1.86)	0.75	1.01 (0.48-2.09)	0.99
Tertile 3	1.95 (1.02-3.72)	0.042	1.55 (0.77-3.09)	0.22	1.71 (0.88-3.32)	0.11
LN (TNF R2)	2.34 (1.20-4.55)	0.012	1.92 (0.92-3.99)	0.08	1.81 (0.91-3.57)	0.090
Interferon-γ						
Tertile 1	1 (reference)		1 (reference)		1 (reference)	
Tertile 2	1.52 (0.74-3.10)	0.25	1.38 (0.68-2.84)	0.38	1.47 (0.72-3.01)	0.29
Tertile 3	1.28 (0.61-2.65)	0.51	0.97 (0.45-2.09)	0.94	1.15 (0.55-2.42)	0.72
LN (Interferon-γ)	1.15 (0.67-1.98)	0.62	0.93 (0.52-1.65)	0.79	1.08 (0.63-1.87)	0.78
IL-6						
Not measurable	1 (reference)		1 (reference)		1 (reference)	
Measurable	0.923 (0.54-1.60)	0.79	1.03 (0.58-1.81)	0.93	0.78 (0.43-1.40)	0.40
IL-8						
Tertile 1	1 (reference)		1 (reference)		1 (reference)	
Tertile 2	0.71 (0.36-1.41)	0.33	0.71 (0.36-1.40)	0.32	0.66 (0.33-1.32)	0.24
Tertile 3	1.00 (0.54-1.86)	1.00	0.95 (0.50-1.80)	0.87	0.83 (0.43-1.58)	0.56
LN (IL-8)	1.25 (0.69-2.27)	0.47	1.18 (0.64-2.17)	0.60	1.07 (0.58-1.97)	0.84
IL-10						
Tertile 1	1 (reference)		1 (reference)		1 (reference)	
Tertile 2	1.28 (0.66-2.48)	0.47	1.31 (0.67-2.57)	0.43	1.12 (0.57-2.20)	0.75
Tertile 3	0.77 (0.36-1.62)	0.48	0.83 (0.38-1.81)	0.65	0.74 (0.35-1.57)	0.43
LN (IL-10)	0.98 (0.72-1.32)	0.88	1.03 (0.75-1.42)	0.87	0.98 (0.71-1.34)	0.89
IL-18						
Tertile 1	1 (reference)		1 (reference)		1 (reference)	
Tertile 2	0.99 (0.49-2.03)	0.98	0.98 (0.48-2.02)	0.96	1.10 (0.53-2.27)	0.81
Tertile 3	1.14 (0.57-2.28)	0.71	1.18 (0.59-2.36)	0.65	1.18 (0.58-2.37)	0.65
LN (IL-18)	1.10 (0.54-2.21)	0.80	1.15 (0.56-2.36)	0.71	1.05 (0.53-2.06)	0.89

[a]Adjusted for age, gender and indication for coronary angiography. [b]Additionally adjusted for diabetes mellitus, hypertension and CRP. Two separate models were constructed for adjustment because of limited number of endpoints.

Supplementary Table 4B. Association of TNF-α, TNF-β, TNF R2, IFN-γ, IL-6, IL-8, IL-10 and IL-18 with occurrence of major adverse cardiac events in patients with stable AP.

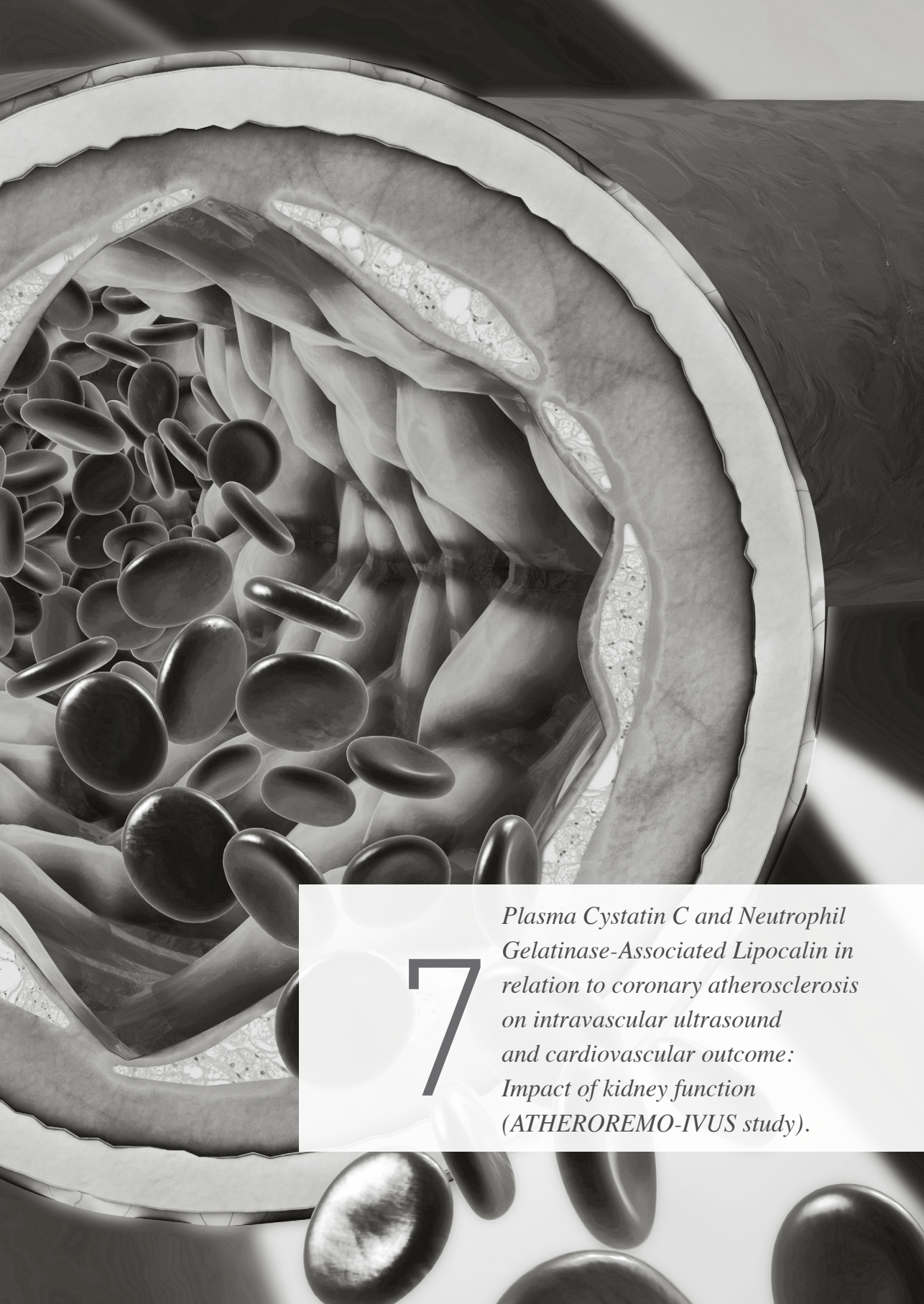
MACE	Unadjusted model		Multivariable model[a]		Multivariable model[b]	
	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
TNF-α						
Tertile 1	1 (reference)		1 (reference)		1 (reference)	
Tertile 2	1.44 (0.55-3.78)	0.46	1.40 (0.53-3.70)	0.50	1.45 (0.55-3.83)	0.46
Tertile 3	0.96 (0.36-2.57)	0.93	0.95 (0.35-2.55)	0.91	0.81 (0.29-2.24)	0.68
TNF-β						
Not measurable	1 (reference)		1 (reference)		1 (reference)	
Measurable	0.33 (0.05-2.46)	0.28	0.35 (0.05-2.55)	0.30	0.34 (0.05-2.47)	0.28
TNF R2						
Tertile 1	1 (reference)		1 (reference)		1 (reference)	
Tertile 2	1.15 (0.40-3.33)	0.79	1.09 (0.37-3.18)	0.88	1.08 (0.37-3.11)	0.89
Tertile 3	2.45 (0.96-6.25)	0.062	2.38 (0.88-6.46)	0.087	2.07 (0.78-5.44)	0.14
LN (TNF R2)	2.99 (1.10-8.13)	0.031	2.80 (0.97-8.07)	0.057	2.29 (0.80-6.53)	0.12
Interferon-γ						
Tertile 1	1 (reference)		1 (reference)		1 (reference)	
Tertile 2	0.97 (0.33-2.89)	0.96	0.93 (0.31-2.76)	0.89	0.94 (0.31-2.82)	0.91
Tertile 3	1.29 (0.48-3.44)	0.61	1.13 (0.41-3.15)	0.82	1.17 (0.43-3.16)	0.76
LN (Interferon-γ)	1.41 (0.68-2.91)	0.36	1.26 (0.59-2.69)	0.56	1.30 (0.62-2.71)	0.49
IL-6						
Not measurable	1 (reference)		1 (reference)		1 (reference)	
Measurable	1.13 (0.51-2.55)	0.76	1.19 (0.53-2.68)	0.67	0.87 (0.36-2.10)	0.76
IL-8						
Tertile 1	1 (reference)		1 (reference)		1 (reference)	
Tertile 2	0.25 (0.08-0.75)	0.014	0.25 (0.08-0.74)	0.012	0.23 (0.07-0.69)	0.009
Tertile 3	0.89 (0.39-2.01)	0.78	0.87 (0.38-1.96)	0.73	0.71 (0.30-1.68)	0.44
LN (IL-8)	1.03 (0.44-2.41)	0.94	0.98 (0.42-2.28)	0.95	0.81 (0.34-1.97)	0.65
IL-10						
Tertile 1	1 (reference)		1 (reference)		1 (reference)	
Tertile 2	1.09 (0.47-2.53)	0.83	1.11 (0.48-2.57)	0.81	1.08 (0.47-2.51)	0.85
Tertile 3	0.62 (0.18-2.21)	0.47	0.60 (0.17-2.12)	0.42	0.50 (0.13-1.90)	0.31
LN (IL-10)	1.28 (0.73-2.27)	0.39	1.26 (0.71-2.22)	0.43	1.17 (0.65-2.14)	0.60
IL-18						
Tertile 1	1 (reference)		1 (reference)		1 (reference)	
Tertile 2	1.33 (0.50-3.57)	0.57	1.32 (0.49-3.55)	0.58	1.20 (0.44-3.27)	0.72
Tertile 3	1.39 (0.52-3.74)	0.51	1.32 (0.49-3.55)	0.58	1.15 (0.42-3.18)	0.78
LN (IL-18)	1.78 (0.61-5.19)	0.29	1.70 (0.58-5.02)	0.33	1.49 (0.50-4.44)	0.48

[a]Adjusted for age, gender and indication for coronary angiography. [b]Additionally adjusted for diabetes mellitus, hypertension and CRP. Two separate models were constructed for adjustment because of limited number of endpoints.

Supplementary Table 4C. Association of TNF-α, TNF-β, TNF R2, IFN-γ, IL-6, IL-8, IL-10 and IL-18 with occurrence of major adverse cardiac events in patients with ACS.

MACE	Unadjusted model		Multivariable model[a]		Multivariable model[b]	
	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
TNF-α						
Tertile 1	1 (reference)		1 (reference)		1 (reference)	
Tertile 2	0.61 (0.22-1.72)	0.35	0.55 (0.20-1.55)	0.26	0.64 (0.22-1.83)	0.40
Tertile 3	0.69 (0.25-1.94)	0.48	0.61 (0.22-1.73)	0.35	0.62 (0.22-1.79)	0.38
TNF-β						
Not measurable	1 (reference)		1 (reference)		1 (reference)	
Measurable	0.95 (0.13-7.02)	0.96	0.96 (0.13-7.09)	0.97	1.01 (0.14-7.52)	0.99
TNF R2						
Tertile 1	1 (reference)		1 (reference)		1 (reference)	
Tertile 2	0.97 (0.35-2.66)	0.95	0.74 (0.26-2.10)	0.57	0.93 (0.33-2.59)	0.89
Tertile 3	1.51 (0.61-3.76)	0.37	1.01 (0.37-2.72)	0.99	1.27 (0.49-3.31)	0.63
LN (TNF R2)	1.95 (0.77-4.96)	0.16	1.39 (0.48-4.00)	0.54	1.41 (0.53-3.74)	0.49
Interferon-γ						
Tertile 1	1 (reference)		1 (reference)		1 (reference)	
Tertile 2	2.06 (0.80-5.32)	0.13	1.92 (0.74-4.97)	0.18	1.90 (0.73-4.94)	0.19
Tertile 3	0.88 (0.26-3.00)	0.83	0.68 (0.19-2.37)	0.54	0.77 (0.22-2.73)	0.69
LN (Interferon-γ)	0.80 (0.35-1.83)	0.60	0.65 (0.28-1.51)	0.32	0.75 (0.32-1.78)	0.52
IL-6						
Not measurable	1 (reference)		1 (reference)		1 (reference)	
Measurable	0.91 (0.42-1.97)	0.82	0.91 (0.42-1.97)	0.81	0.73 (0.32-1.70)	0.47
IL-8						
Tertile 1	1 (reference)		1 (reference)		1 (reference)	
Tertile 2	2.49 (0.78-7.94)	0.12	2.36 (0.74-7.58)	0.15	2.37 (0.74-7.59)	0.15
Tertile 3	1.94 (0.62-6.09)	0.26	1.48 (0.46-4.80)	0.51	1.56 (0.48-5.07)	0.46
LN (IL-8)	1.70 (0.71-4.06)	0.23	1.38 (0.56-3.41)	0.49	1.43 (0.58-3.51)	0.43
IL-10						
Tertile 1	1 (reference)		1 (reference)		1 (reference)	
Tertile 2	1.88 (0.58-6.11)	0.29	1.81 (0.55-5.97)	0.33	1.51 (0.45-5.04)	0.51
Tertile 3	1.20 (0.37-3.89)	0.76	1.20 (0.37-3.90)	0.77	1.10 (0.34-3.60)	0.88
LN (IL-10)	0.95 (0.63-1.42)	0.80	0.96 (0.64-1.43)	0.84	0.95 (0.63-1.44)	0.81
IL-18						
Tertile 1	1 (reference)		1 (reference)		1 (reference)	
Tertile 2	0.72 (0.25-2.08)	0.55	0.67 (0.23-1.96)	0.47	0.94 (0.31-2.81)	0.91
Tertile 3	0.95 (0.36-2.54)	0.92	1.04 (0.39-2.77)	0.94	1.09 (0.40-2.96)	0.87
LN (IL-18)	0.77 (0.29-2.00)	0.58	0.82 (0.30-2.23)	0.70	0.76 (0.31-1.89)	0.56

[a]Adjusted for age, gender and indication for coronary angiography. [b]Additionally adjusted for diabetes mellitus, hypertension and CRP. Two separate models were constructed for adjustment because of limited number of endpoints.



7

Plasma Cystatin C and Neutrophil Gelatinase-Associated Lipocalin in relation to coronary atherosclerosis on intravascular ultrasound and cardiovascular outcome: Impact of kidney function (ATHEROREMO-IVUS study).

7

Plasma Cystatin C and Neutrophil Gelatinase-Associated Lipocalin in relation to coronary atherosclerosis on intravascular ultrasound and cardiovascular outcome: Impact of kidney function (ATHEROREMO-IVUS study).

Brankovic M, Akkerhuis KM, Buljubasic N, Cheng JM, Oemrawsingh RM, Garcia-Garcia HM, Regar E, Serruys PW, van Geuns RJ, Boersma E, Kardys I.

Atherosclerosis 2016; 254: 20-27. doi: 10.1016/j.atherosclerosis.2016.09.016.

Abstract

Rationale: We investigated whether plasma cystatin C (CysC) and neutrophil gelatinase-associated lipocalin (NGAL) are associated with intravascular ultrasound (IVUS)-derived characteristics of coronary atherosclerosis and 1-year adverse coronary events in patients with normal and mildly-to-moderately impaired kidney function.

Methods & Results: Between 2008 and 2011, virtual histology (VH)-IVUS of a non-culprit coronary artery was performed in 581 patients undergoing coronary angiography. Creatinine, CysC and NGAL were measured in pre-procedural blood samples. Presence of VH-IVUS-derived thin-cap fibroatheroma (TCFA) lesions, lesions with plaque burden (PB) ≥70% and lesions with minimal luminal area (MLA) ≤4mm^2 was assessed. Major adverse coronary events (MACE) comprised the composite of all-cause mortality, acute coronary syndrome, or unplanned coronary revascularization. Analyses were stratified using $eGFR_{Cr}$

of 90 ml/min/1.73m^2 as the cut-off. In patients with normal kidney function, those with higher CysC levels had fewer lesions with PB ≥70% and fewer VH-TCFA lesions (adjusted odds ratios (ORs) and 95% confidence intervals (CIs): 0.46 (0.30-0.69) and 0.59 (0.44-0.83), respectively, per standard deviation (SD) ln (ng/mL) CysC). Those with higher NGAL levels also had fewer lesions with PB ≥70% (adjusted OR (95% CI): 0.49 (0.29-0.82)) in patients with impaired kidneys, no differences in high-risk lesions were observed for CysC or NGAL. However, those with higher CysC had higher risk of MACE (hazard ratio (HR): 1.4, 95% CI (1.03-1.92)). This was not the case in patients with normal kidney function. NGAL did not influence risk of MACE.

Conclusions: Mild-to-moderate kidney dysfunction modifies the relationship between CysC and high-risk coronary lesions. This has not been established before, and offers an explanation for the difference in findings between experimental and epidemiologic studies.

Introduction

Kidney impairment, as assessed by creatinine-based equations of glomerular filtration rate ($eGFR_{Cr}$), is associated with cardiovascular disease independently of established cardiovascular risk factors [1]. In persons with mild kidney dysfunction ($eGFR_{Cr}$ in the range of 60-89 ml/min/1.73m^2), cystatin C (CysC) may outperform $eGFR_{Cr}$ as a predictor of adverse outcome. This is illustrated by the fact that CysC displays a linear association with mortality in patients with such mild GFR reduction, while $eGFR_{Cr}$ has a J-shaped association with mortality, and risk only starts to rise when $eGFR_{Cr}$ falls beneath 60 ml/min/1.73m^2 [2,3]. Although some studies have shown linear associations of $eGFR_{Cr}$ with adverse outcome, these associations were linear only in particular ranges of $eGFR_{Cr}$ (specifically, $eGFR_{Cr}$ above 60) [4].

CysC is a cysteine protease inhibitor produced by most nucleated cells, and can be detected in serum or plasma [5]. In in vitro and animal experiments, a reduction of CysC correlated with increased activity of cysteine proteases cathepsins K and S, which led to breakdown of the elastic lamina in the blood vessel wall [6]. Altered CysC expression has been identified in diseases which progress by extracellular proteolysis, such as atherosclerosis and aortic aneurysms, and metastasis [7,8]. These experiments, pointing towards a favourable role for CysC, do not concur with the positive associations of CysC with adverse outcomes

found in epidemiological studies. Studies on the in-vivo association between plasma CysC and coronary atherosclerosis may provide further insight into this discrepancy, but have not yet been performed.

Neutrophil gelatinase-associated lipocalin (NGAL) is a clinically relevant biomarker in acute kidney injury [9] due to its marked increase in plasma and urine after tubulo-interstitial kidney damage [10]. Recently, overexpression of plasma NGAL has been found in coronary plaques, where NGAL inhibits elimination of matrix metalloproteinase-9 (MMP-9) [11,12]. MMP-9 is involved in extracellular matrix degradation, herewith increasing the risk of plaque rupture [13]. NGAL and NGAL/MMP-9 complex have been shown to predict major adverse cardiovascular events in epidemiological studies [14,15].

In spite of the above-described associations that have been demonstrated between CysC, NGAL and adverse cardiac events, the presence and shape of a relationship between plasma CysC, NGAL, and coronary atherosclerosis have not yet been investigated *in vivo*. To the best of our knowledge, we are the first to perform such an investigation, and to herewith provide a link between fundamental experiments and epidemiological studies. Specifically, our study aimed to investigate whether plasma CysC and NGAL are associated with IVUS-derived characteristics of in-vivo coronary atherosclerosis and 1-year adverse coronary events in patients with normal and mildly-to-moderately impaired kidney function.

Materials and methods

Study population

We have previously described the design of The European Collaborative Project on Inflammation and Vascular Wall Remodeling in Atherosclerosis - Intravascular Ultrasound (ATHEROREMOIVUS) [16]. In this study, we included 581 patients undergoing diagnostic coronary angiography or percutaneous coronary intervention (PCI) for acute coronary syndrome (ACS) or stable angina pectoris (SAP) between 2008 and 2011 in the Erasmus MC, Rotterdam, the Netherlands. Following coronary angiography, intravascular ultrasound (IVUS) of a non-culprit coronary artery was performed. The human research ethics committee of Erasmus MC, Rotterdam, the Netherlands has approved this study. All included patients have signed informed consent, and the study protocol conformed to the Declaration of Helsinki. This study is registered in ClinicalTrials.gov (number: NCT01789411).

Kidney function assessment

Estimated Glomerular Filtration Rate ($eGFR_{Cr}$) was assessed by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [17]. Patients were categorized according to eGFR by using the modified definition from the National Kidney Foundation e Kidney Disease Outcome Quality Initiative (K/DOQI) clinical practice guidelines [18]: normal (GFR≥90 ml/min/1.73m^2), mild (GFR 60-89 ml/min/1.73m^2), moderate (GFR 30-59 ml/min/1.73m^2), and severe (GFR 15-29 ml/min/1.73m^2) kidney dysfunction, and kidney failure (GFR<15 ml/min/1.73m^2). No patients with kidney failure were present in this study, and only one patient had $eGFR_{Cr}$<30 ml/min/1.73m^2. The latter was excluded from further analyses. Patients were stratified into those with normal kidney function and those with mildly-to-moderately impaired kidney function, using an $eGFR_{Cr}$ of 90 ml/min/1.73m^2 as the cut-off value.

Biomarkers

Arterial blood was taken before the procedure and stored at -80 °C within 2h. Samples were available in 570 patients. An immunoturbidimetric high sensitivity assay (Roche Diagnostics Ltd., Rotkreuz, Switzerland) on the Roche Cobas 8000 modular analyzer platform was used in the Erasmus MC clinical laboratory to measure the level of C-reactive protein (CRP) in serum samples. The plasma EDTA samples were transported at a temperature of -80 °C to Myriad RBM, Austin, Texas, USA, where cystatin C and NGAL concentrations were assessed by a validated multiplex assay (Custom Human Map, Myriad RBM, Austin, Texas, USA). As a result of the batch-wise handling of the samples, with an update of the composition of the multiplex assay by the manufacturer in between two batches, cystatin C was measured in the full cohort of 570 patients, and NGAL in a random subset of 473 patients. Both laboratories were blinded to clinical and imaging data.

Grayscale and radiofrequency intravascular ultrasound (IVUS)

Following coronary angiography, we performed IVUS imaging of the most proximal part of a non-culprit, non-treated coronary vessel. The non-culprit vessel was selected based on the following order: left anterior descending artery; right coronary artery; left circumflex artery. We obtained all IVUS data by the Volcano s5/s5i Imaging System (Volcano Corp., San Diego, CA, USA) using a Volcano-Eagle-Eye Gold IVUS catheter of 20 MHz with an automatic pullback system and a standard pullback speed of 0.5mm/s.

Subsequently, an independent core laboratory (Cardialysis BV, Rotterdam, the

Netherlands) analysed IVUS images offline, blinded for clinical and biomarker data. The IVUS virtual histology (IVUSVH) was assessed by pcVH 2.1 and qVH (Volcano Corp., San Diego, CA, USA) software. In each frame, the external elastic membrane and luminal borders were outlined (median interslice distance, 0.40mm).

The degree (plaque volume and plaque burden) and composition of the atherosclerotic plaque were assessed. Plaque volume was defined as the total volume of the external elastic membrane occupied by atheroma [19]. Plaque burden was defined as the plaque and media cross-sectional area divided by the external elastic membrane cross-sectional area and is presented as a percentage. A coronary lesion as defined as a segment with a plaque burden of more than 40% in at least three consecutive frames [16]. The composition of the atherosclerotic plaque was characterized into fibrous, fibro-fatty, dense calcium and necrotic core [20]. Subsequently, three types of VH-IVUS high-risk lesions were identified: 1. Thin-cap fibroatheroma (TCFA) lesion: a lesion with the presence of >10% confluent necrotic core in direct contact with the lumen; 2. A lesion with a plaque burden of ≥70%; 3. a lesion with a minimal luminal area (MLA) of ≤4.0mm^2 [21].

Follow-up

Clinical follow-up started at inclusion and lasted one year. The primary clinical endpoint - MACE - was the composite of all-cause mortality, ACS, or unplanned coronary revascularization. ACS was defined as the clinical diagnosis of ST-segment elevation myocardial infarction (STEMI), non-STEMI, or unstable angina pectoris using the guidelines of the European Society of Cardiology [22,23]. Unplanned coronary revascularizations were defined as unplanned coronary artery bypass grafting or repeat percutaneous coronary intervention. The secondary endpoint was the composite of all-cause mortality or ACS. The endpoints were adjudicated by a clinical event committee blinded for biomarker and IVUS data.

Statistical analysis

The Kolmogorov-Smirnov test was used to test distributions of continuous variables for normality. CysC and CRP were not normally distributed and were ln-transformed for further analyses. Categorical variables are presented as numbers and percentages. Continuous variables that were normally distributed are presented as mean ± standard deviation (SD); non-normally distributed continuous variables are presented as median and interquartile range [IQR]. For reasons of uniformity, all biomarkers are presented as median [IQR].

We examined the associations of plasma CysC and NGAL levels with plaque burden, plaque volume, and the presence of high-risk coronary lesions. Plaque volume was normalized for the imaged segment length. We used linear regression and logistic regression analyses with continuous ln transformed CysC and NGAL concentrations consecutively as independent variables. To assess the effect of kidney function, we included interaction terms (ln-transformed CysC or NGAL, respectively, with dichotomized $eGFR_{Cr}$ (above or below 90 ml/min/1.73m^2)) into the logistic regression models. Subsequently, we stratified all analyses on $eGFR_{Cr}$ of 90 ml/min/1.73m^2. To test whether effect estimates differed between patients with ACS and patients with SAP, Z-tests for heterogeneity were performed.

Cox proportional hazards regression analyses were performed to evaluate the associations between CysC and NGAL and the clinical study endpoints.

Age, gender, indication for coronary angiography, diabetes mellitus, hypertension, and CRP concentration were considered as potential confounders, and were therefore entered into the multivariable linear and logistic regression models. Multivariable adjustment of Cox proportional hazards models was constrained due to the number of clinical endpoints, and was therefore performed in two steps. For MACE, in the first step the adjustment included age, gender, and indication for angiography; in the second step, diabetes mellitus, hypertension and CRP were added.

Finally, we determined the cut-off values of CysC and NGAL that carry the optimal discriminative ability with respect to presence of high-risk coronary lesions and occurrence of MACE. For this purpose, we drew receiver operating characteristic (ROC) curves and calculated the Youden index (highest sum of sensitivity and specificity -1) [24]. We considered only statistically significant associations.

All data were analysed with SPSS software (SPSS 20.0; IBM Corp., Armonk, NY). All statistical tests were two tailed, and p values <0.05 were considered statistically significant.

Results

Baseline characteristics

Mean age was 61.6 ± 11.4 years, 75.7% were men, 54.6% had ACS, and 45.4% had SAP (Table 1). The imaged coronary segment had a median length of 44.3 [33.8-55.4] mm. A total of 239 (41.5%) patients had at least one TCFA lesion, 120 (21.0%) had lesions with PB ≥70%, and 175

(30.7%) had lesions with MLA≤4mm^2. Median eGFR$_{Cr}$ was 90 [77-98] ml/min/1.73mm^2 in the full cohort with similar values in the subset of ACS patients (91 [78-100] ml/min/ 1.73mm^2) and SAP patients (89 [77-97] ml/min/1.73mm^2). A total of 291 (51.8%) patients had normal kidney function and 271 (48.2%) patients had mild-to-moderate kidney dysfunction. ACS patients exhibited significantly higher NGAL levels compared to patients with SAP, regardless of kidney function, whereas plasma CysC levels were similar in both eGFR$_{Cr}$ groups (Table 1).

Table 1. Baseline characteristics.

	Total (n = 570)	ACS patients (n = 309)	SAP patients (n = 261)
Patient characteristics			
Age, years	61.5 ± 11.4	59.7 ± 11.9	63.6 ± 10.3
Male gender, n (%)	430 (75.4)	227 (73.5)	203 (77.8)
Hypertension, n (%)	295 (51.8)	134 (43.4)	161 (61.7)
Hypercholesterolemia, n (%)	317 (55.6)	137 (44.3)	180 (69.0)
Diabetes mellitus, n (%)	99 (17.4)	40 (12.9)	59 (22.6)
Positive family history, n (%)	293 (51.4)	140 (45.3)	153 (58.6)
Smoking, n (%)	164 (28.8)	115 (37.2)	49 (18.8)
Peripheral artery disease, n (%)	36 (6.3)	12 (3.9)	24 (9.2)
Previous myocardial infarction, n (%)	184 (32.3)	80 (25.9)	104 (39.8)
Previous PCI, n (%)	185 (32.5)	57 (18.4)	128 (49.0)
Previous CABG, n (%)	18 (3.2)	7 (2.3)	11 (4.2)
Previous stroke, n (%)	23 (4.0)	10 (3.2)	13 (5.0)
History of heart failure, n (%)	19 (3.3)	6 (1.9)	13 (5.0)
Procedural characteristics			
PCI performed, n (%)	501 (87.9)	287 (92.9)	214 (82.0)
Indication for coronary angiography			
Acute coronary syndrome, n (%)	309 (54.2)	309 (100)	0 (0)
Myocardial infarction, n (%)	159 (27.9)	159 (51.5)	0 (0)
Unstable angina pectoris, n (%)	150 (26.3)	150 (48.5)	0 (0)
Stable angina pectoris, n (%)	261 (45.8)	0 (0)	261 (100)
Coronary artery disease [a]			
No significant stenosis, n (%)	42 (7.4)	18 (5.8)	24 (9.2)
1-vessel disease, n (%)	301 (52.8)	168 (54.4)	133 (51.0)
2-vessel disease, n (%)	166 (29.1)	88 (28.5)	78 (29.9)
3-vessel disease, n (%)	61 (10.7)	35 (11.3)	26 (10.0)
IVUS segment characteristics			
Segment length, mm	44.1 [33.7-55.4]	43.9 [32.9-54.1]	44.8 [34.2-57.2]
Plaque burden, %	39.2 [30.0-46.4]	37.2 [28.0-45.5]	40.2 [31.8-47.8]
Presence of VH-TCFA, n (%)	239 (41.9)	140 (45.3)	99 (37.9)
Presence of PB ≥70%, n (%)	120 (21.0)	56 (18.1)	64 (24.5)
Presence of MLA ≤4mm^2, n (%)	175 (30.7)	87 (28.2)	88 (33.7)
Renal function			
eGFR[b,c], ml/min/1.73 m^2	90 [77-98]	91 [78-100]	89 [77-97]

Table 1 continued.

		Total (n = 570)	ACS patients (n = 309)	SAP patients (n = 261)
Renal function				
KDOQI classification, n (%)	GFR ≥90 ml/min/1.73 m^2	291 (51.8)	165 (54.3)	126 (48.8)
	GFR 60-89 ml/min/1.73 m^2	231(41.1)	115 (37.8)	116 (45.0)
	GFR 30-59 ml/min/1.73 m^2	39 (6.9)	23 (7.6)	16 (6.2)
	GFR <30 ml/min/1.73 m^2	1 (0.1)	1 (0.3)	0 (0.0)
Serum biomarkers				
NGAL, ng/mL[d]		197.0 [143.0-254.0]	204.0 [148.2-274.5]	177.0 [141.5-239.0]
GFR_{Cr} ≥90 ml/min/1.73 m^2[e]		183.0 [143.0-227.0]	193.0 [143.0-243.0]	174.0 [125.0-223.0]
GFR_{Cr} 30-89 ml/min/1.73 m^2[e]		216.0 [148.0-293.2]	228.5 [149.0-307.0]	197.0 [143.5-257.7]
Cystatin C, ng/mL		796.0 [691.0-923.0]	791.0 [674.5-915.5]	802.0 [712.5-935.5]
GFR_{Cr} ≥90 ml/min/1.73 m^2		732.0 [644.0-834.0]	729.0 [637.5-841.5]	734.5 [650.7-822.5]
GFR_{Cr} 30-89 ml/min/1.73 m^2		872.0 [775.7-1032.5]	863.0 [745.0-1040.0]	879.0 [781.0-1030.0]
Creatinine, umol/L		77 [66-86]	77 [65-877]	76 [67-86]
C-reactive protein, mg/L		2.1 [0.8-5.3]	2.8 [1.1-6.9]	1.4 [0.6-3.1]

ACS=acute coronary syndrome; SAP=stable angina pectoris; CABG=coronary artery bypass grafting; PCI=percutaneous coronary intervention; CKD=chronic kidney disease; NGAL=neutrophil gelatinase-associated lipocalin; PB=plaque burden; MLA=minimal luminal area. [a]Significant stenosis was defined as a stenosis >50% of the vessel diameter by visual assessment on the coronary angiogram. [b]Estimated Glomerular Filtration Rate ($eGFR_{Cr}$) using CKD-EPI equation: *$GFR = 141 \times min(Scr/\varkappa, 1)^{\alpha} \times max(Scr/\varkappa, 1)^{-1.209} \times 0.993^{Age} \times 1.018$ [if female] $\times$ 1.159 [if black] where: Scr is serum creatinine in mg/dL, $\varkappa$ is 0.7 for females and 0.9 for males, α is -0.329 for females and -0.411 for males, min indicates the minimum of Scr /$\varkappa$ or 1, and max indicates the maximum of Scr /$\varkappa$ or 1.* [c]Creatinine available in 99%, Total n=562, ACS n=304, SAP n=258. [d]Measurable in sample of total n=473, ACS n=257, SAP n=216. [e]A statistically significant difference in plasma NGAL levels between ACS and SAP patients (for total population, p=0.002; if $eGFR_{Cr}$ ≥90 ml/min/1.73 m^2, p=0.01; if $eGFR_{Cr}$ 30-89 ml/min/1.73 m^2, p=0.03).

Cystatin C, NGAL and degree of atherosclerosis on grayscale IVUS

Numbers of lesions with plaque burden (PB) ≥70% and minimal luminal area (MLA) ≤4mm^2 according to categories of kidney function are depicted in Supplementary Figure 3. Significant interactions were found between CysC and $eGFR_{Cr}$ in crude (p=0.007) and multivariable (p=0.010) models predicting lesions with PB ≥70%. In patients with normal kidney function, those with higher CysC had lower risk of lesions with PB ≥70% (per SD increase in ln-transformed CysC: OR (95% CI): 0.56 (0.39-0.82), p=0.002) (Table 2, Figure 1A, 1C, Supplementary Figure 1). After multivariable adjustment including CRP levels, risk remained significantly lower (adjusted OR (95% CI): 0.46 (0.30-0.69), p<0.001). A CysC level of 773.0 ng/ml was the optimal cut-off value to identify patients who did not have lesions with PB ≥70% (CysC ≥773.0 ng/ml) (Supplementary Figure 4). Conversely, in patients with mild-to-moderate kidney dysfunction risk did not differ significantly according to CysC levels (adjusted OR (95% CI): 0.95 (0.69-1.30), p=0.75). Risk of lesions with PB

≥70% displayed a similar pattern in patients with higher NGAL (Table 2). In patients with normal kidney function, an NGAL level of 180.0 ng/ml was the optimal cutoff value to identify patients without lesions with PB ≥70% (NGAL ≥180.0 ng/ml) (Supplementary Figure 5). Risk of lesions with MLA ≤4mm^2 was not different for patients with higher CysC or NGAL (Table 2).

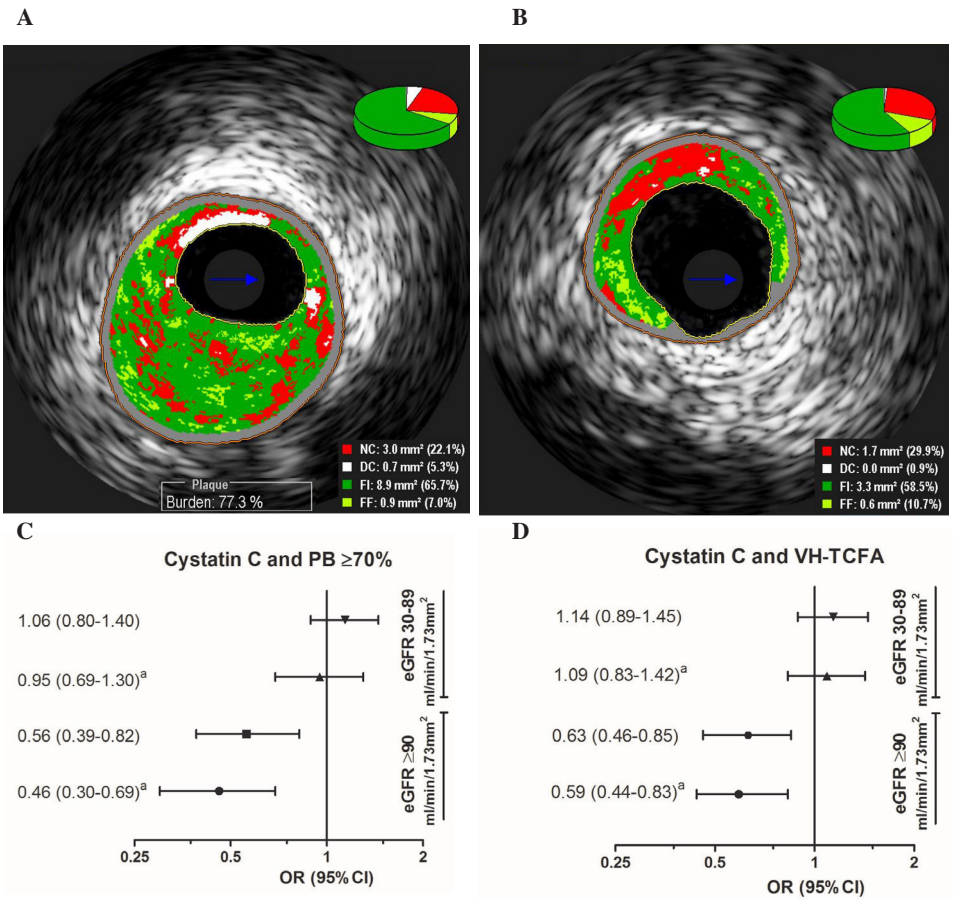


Figure 1. Plasma cystatin C and presence of VH-IVUS high-risk coronary lesions.

Images: courtesy of J. Ligthart and K. Witberg, Invasive Imaging, Dpt. of Cardiology, Erasmus MC. FI=fibrous; FF=fibro-fatty; NC=necrotic core; DC=dense calcium. [a]Adjusted for age, gender, diabetes, hypertension, indication for angiography, C-reactive protein. (A) Lesion with plaque burden (PB) ≥70%. Plaque burden is defined as plaque and media cross-sectional area (i.e., area between yellow contour and red contour) divided by external elastic membrane cross-sectional area (contoured in red); (B) VH-IVUS derived thin-cap fibroatheroma lesion (VH-TCFA), defined as a lesion (i.e., plaque with a plaque burden >40%) with presence of confluent necrotic core >10% in direct contact with the lumen in at least three frames; (C) Odds ratio (OR) per standard deviation increase in ln-transformed cystatin C with 95% confidence interval (CI) for lesions with PB ≥70%. (D) Odds ratio (OR) per standard deviation increase in ln-transformed cystatin C with 95% confidence interval (CI) for VH-TCFA lesions.

Table 2. Plasma cystatin C, NGAL and presence of thin-cap fibroatheroma (VH-TCFA) lesions, lesions with plaque burden (PB) ≥70% and lesions with minimal luminal area (MLA) ≤4mm² stratified according to kidney function ($eGFR_{Cr}$).

	Unadjusted model	P	Multivariable model[c]	P
	OR (95% CI)		OR (95% CI)	
$eGFR_{Cr}$ ≥ 90 mL/min/1.73 m²				
VH-TCFA				
Cystatin C[a]	0.63 (0.46-0.85)	0.002	0.59 (0.44-0.83)	0.002
NGAL[b]	0.77 (0.57-1.04)	0.090	0.72 (0.52-0.98)	0.040
Plaque burden ≥70%				
Cystatin C[a]	0.56 (0.39-0.82)	0.002	0.46 (0.30-0.69)	<0.001
NGAL[b]	0.56 (0.35-0.89)	0.015	0.49 (0.29-0.82)	0.007
MLA ≤4mm²				
Cystatin C[a]	0.97 (0.72-1.32)	0.88	0.92 (0.67-1.25)	0.59
NGAL[b]	1.03 (0.77-1.37)	0.84	1.07 (0.79-1.45)	0.67
$eGFR_{Cr}$ 30-89 mL/min/1.73 m²				
VH-TCFA				
Cystatin C[a]	1.14 (0.89-1.45)	0.30	1.09 (0.83-1.42)	0.55
NGAL[b]	1.01 (0.78-1.29)	0.97	0.96 (0.74-1.24)	0.74
Plaque burden ≥70%				
Cystatin C[a]	1.06 (0.80-1.40)	0.68	0.95 (0.69-1.30)	0.75
NGAL[b]	1.20 (0.88-1.63)	0.25	1.21 (0.87-1.67)	0.25
MLA ≤4mm²				
Cystatin C[a]	0.84 (0.64-1.10)	0.19	0.73 (0.53-0.99)	0.042
NGAL[b]	0.98 (0.74-1.29)	0.90	1.05 (0.78-1.41)	0.75

[a] Odds ratio (OR) per standard deviation increase in ln-transformed cystatin C with 95% confidence interval (CI). [b]Odds ratio (OR) per standard deviation increase in NGAL with 95% confidence interval (CI). [c]Multivariable model: adjusted for age, gender, diabetes mellitus, hypertension, indication for angiography, C-reactive protein.

Overall, no differences could be demonstrated between CysC and NGAL in either plaque burden or normalized plaque volume of the entirely imaged segment (Table 3 and Supplementary Table 2). Nevertheless, CysC showed a tendency towards lower normalized segment plaque volume (per SD increase in ln-transformed CysC: β (95% CI): -0.43 (-1.02-0.16), p=0.16) in patients with normal kidney function; whereas no differences were observed in patients with mild-to-moderate kidney dysfunction.

There was no heterogeneity between ACS and SAP patients regarding the differences in IVUS grayscale parameters according to CysC or NGAL levels.

Table 3. Plasma cystatin C, NGAL and segment characteristics (degree of atherosclerosis: plaque volume and plaque burden; composition of coronary atherosclerosis: 4 components) as determined by VH-IVUS stratified according to kidney function ($eGFR_{Cr}$).

	Cystatin C[b]	P	NGAL[c]	P
	β (95% CI)		β (95% CI)	
$eGFR_{Cr}$ ≥ 90 mL/min/1.73 m²				
Plaque burden[a]	-0.02 (-0.16-0.12)	0.77	-0.05 (-0.18-0.09)	0.50
Plaque volume[a]	-0.43 (-1.02-0.16)	0.16	-0.19 (-0.77-0.38)	0.51
Fibrous, %	0.52 (-1.11-2.15)	0.53	0.60 (-0.98-2.19)	0.45
Fibro-fatty, %[a]	0.03 (-1.10-0.17)	0.65	0.12 (-0.02-0.25)	0.09
Necrotic core, %	-0.65 (-1.84-0.53)	0.28	-0.85 (-2.00-0.30)	0.15
Dense calcium, %[a]	0.00 (-0.17-0.17)	0.99	-0.12 (-0.28-0.04)	0.15
$eGFR_{Cr}$ 30-89 mL/min/1.73 m²				
Plaque burden[a]	0.00 (-0.11-0.12)	0.94	-0.03 (-0.15-0.09)	0.66
Plaque volume[a]	0.16 (-0.37-0.68)	0.55	-0.04 (-0.59-0.51)	0.89
Fibrous, %	-1.04 (-2.45-0.37)	0.15	0.60 (-0.89-2.09)	0.42
Fibro-fatty, %[a]	-0.02 (-0.13-0.10)	0.76	-0.01 (-0.12-0.11	0.92
Necrotic core, %	0.44 (-0.47-1.35)	0.34	-0.27 (-1.23-0.68)	0.57
Dense calcium, %[a]	0.11 (-0.04-0.25)	0.15	-0.06 (-0.21-0.09)	0.44

[a]Square root transformed. [b]Unadjusted b coefficient per standard deviation increase in ln-transformed cystatin C with 95% confidence interval (CI). [c]Unadjusted b coefficient per standard deviation increase in NGAL with 95% confidence interval (CI).

Cystatin C, NGAL and composition of atherosclerosis on radiofrequency VH-IVUS

Numbers of thin-cap fibroatheroma lesions (VH-TCFAs) according to categories of kidney function are depicted in Supplementary Figure 3. Significant interactions were found between CysC and $eGFR_{Cr}$ in crude (p=0.002) and multivariable (p=0.003) models predicting VH-TCFAs. In patients with normal kidney function, those with higher CysC levels had lower risk of VH-TCFA lesions (per SD increase in ln-transformed CysC: OR (95% CI): 0.63 (0.46-0.85), p=0.002) (Table 2, Figure 1B, 1D, Supplementary Figure 1). After multivariable adjustment including CRP levels, risk remained significantly lower (adjusted OR (95% CI): 0.59 (0.44-0.83), p=0.002). CysC of 678.5 ng/mL was the optimal cut-off value to identify patients without VH-TCFA lesions (CysC ≥678.5 ng/mL) (Supplementary Figure 6). Conversely, in patients with mild-to-moderate kidney dysfunction, risk did not differ significantly according to CysC levels (adjusted OR (95% CI): 1.09 (0.83-1.42), p=0.55). The interaction between NGAL and eGFRCr was not statistically significant. A tendency towards lower risk of VH-TCFA lesions was observed for higher NGAL, but only in patients with normal kidney function (Table 2). There was no heterogeneity between ACS and SAP patients regarding the difference in VH-TCFA lesions (CysC, p=0.29, NGAL, p=0.57) (Supplementary Table 1).

At the level of the entire segment, no differences were present in radiofrequency VH-tissue types between CysC or NGAL (Table 3 and Supplementary Table 2).

Cystatin C, NGAL and 1-year MACE

Vital status was acquired for 569 (99.8%) patients. During the 1-year follow-up, 56 patients experienced the primary endpoint (MACE; Supplementary Figure 3), and 30 patients endured the secondary composite endpoint of all-cause mortality or ACS. In the full cohort, patients with higher CysC had higher risk of MACE (per SD increase in ln-transformed CysC: HR (95% CI):1.41 (1.10-1.79), p=0.006) (Figure 2, Supplementary Figure 2). After multivariable adjustment, the risk estimate lost statistical significance. For NGAL, significant differences in risk of MACE were not found (Figure 2, Supplementary Figure 2).

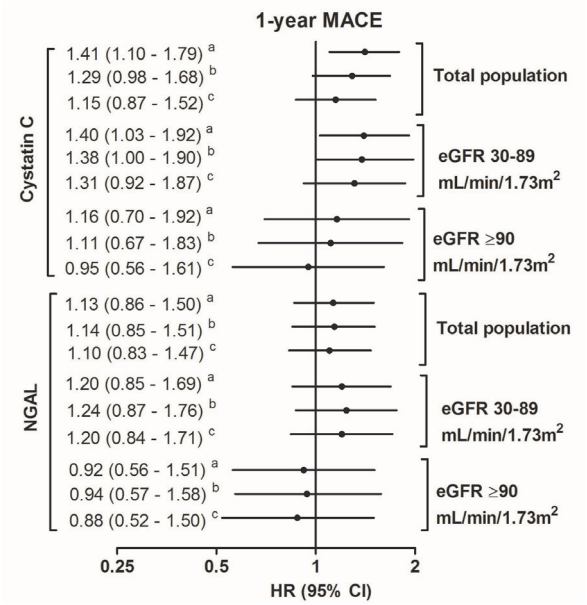


Figure 2. Plasma cystatin C, NGAL and occurrence of the 1-year MACE.

MACE=major adverse coronary event. Hazard ratio (HR) per standard deviation increase in ln-transformed cystatin C and per standard deviation increase in NGAL with 95% confidence interval (CI). [a]Unadjusted model. [b]Adjusted for age, gender, indication for angiography. [c]Adjusted for age, gender, indication for angiography, diabetes mellitus, hypertension, C-reactive protein; multivariable adjustment was constrained by the limited number of clinical endpoints.

In patients with normal kidney function, those with higher CysC levels did not have higher risk of MACE. (Figure 2, Supplementary Figure 2). In patients with mild-to-moderate

kidney dysfunction, those with higher CysC levels had higher risk of MACE in univariable analysis (HR (95% CI): 1.40 (1.03-1.92), p=0.03) (Figure 2, Supplementary Figure 2). In multivariable analysis, the HR lost statistical significance, but did not materially change (HR (95% CI): 1.31 (0.92-1.87), p=0.12).

Both in the total population and in patients with mild-to-moderate kidney dysfunction, a CysC of 849.0 ng/mL was the optimal cut-off value to identify patients who developed MACE (CysC ≥849.0 ng/mL) (Supplementary Figure 7).

Patterns of risk of the secondary endpoint (all-cause mortality and ACS) according to CysC and NGAL levels were similar to those of MACE (Supplementary Table 3).

Finally, stratification on the indication for angiography confirmed the risk patterns which were found in the full cohort (Supplementary Table 4).

Discussion

We found that in patients with normal kidney function, those with higher CysC levels had fewer high-risk coronary lesions (VH-TCFA and lesions with PB ≥70%), while risk of MACE was not different. Conversely, when kidney function was mildly-to-moderately impaired, no differences in high-risk lesions were observed, but those with higher CysC levels had higher risk of MACE. Therefore, with regard to prediction of cardiovascular risk, CysC appears to carry potential only when $eGFR_{Cr}$ is below 90 mL/min/1.73m^2. Furthermore, patients with higher NGAL levels had fewer lesions with PB ≥70%, but only when they had normal kidney function. No differences in MACE were found for NGAL, and thus its use for cardiovascular risk prediction could not be substantiated. Altogether, our results on CysC suggest novel pathophysiological insights, because they offer an explanation for the difference in findings observed in experimental and epidemiologic studies so far, and imply that the association between CysC and cardiovascular disease may not be solely explained through its correlation with GFR.

Higher CysC levels have been associated with occurrence of cardiovascular events in various epidemiological studies [25]. Conversely, animal experiments suggest that higher CysC may be favourable. Atherosclerotic mice deficient in CysC display increased plaque size and macrophage content, increased elastic lamina degradation and accumulation of smooth muscle cells [26,6]. Studies in humans have also found reduced CysC in atherosclerotic

and aneurysmatic aortic lesions [7]. Xu et al. have demonstrated that immune cells (CD8þ dendritic cells (DC) and macrophages), which are involved in atherosclerotic processes, are major contributors to the circulating CysC pool [27,28]. However, besides a correlation with GFR, the mechanisms that may explain the link between CysC and cardiovascular disease are still unclear. Our study provides additional insights. We found that in patients with normal kidney function, those with higher CysC levels had fewer high-risk coronary lesions, and did not have higher risk of MACE. This is in accordance with a potential 'atheroprotective' effect.

Conversely, in patients with mild-to-moderate kidney dysfunction, differences in high-risk lesions according to CysC level were not present. This could possibly be explained by the changes in CysC physiology that occur in impaired kidneys. When kidney function deteriorates, circulating plasma CysC increases and oxidative stress advances, both of which stimulate Cys to form homodimers [28,29]. When CysC forms homodimers, it cannot inhibit cysteine proteases, because the inhibitory region is hidden within the dimer interface. Thus, it may no longer be able to exhibit 'athero-protective' properties [30]. Although these hypotheses are compelling, additional clinical and experimental studies are necessary to further substantiate the effect modification by kidney function that we observed.

Our findings suggest that NGAL may act on coronary artery disease through a different mechanism than currently investigated. A potential lack of predictive precision due to a limited number of MACE may explain the difference between the current results and previous studies [15,31]. On the other hand, a recent meta-analysis that investigated NGAL as a predictor of cardiovascular disease concluded that strong evidence for independent predictive value of NGAL is still lacking [32]. Notably, we found higher plasma NGAL levels in ACS patients compared to SAP patients, independently of kidney function. This could possibly be explained by neutrophilia as a consequence of more severe cardiac damage in ACS patients compared to SAP patients [33]. However, no heterogeneity between ACS and SAP patients was observed in the relationship between NGAL and IVUS-features of coronary atherosclerosis. Some limitations of this study merit consideration. This study is currently the largest cohort in which the associations between IVUS plaque characteristics, CysC and NGAL were investigated. Yet, we cannot exclude the possibility of a chance finding with regard to effect modification by kidney function. However, both the cut-off value (based on K/DOQI guidelines) and the study population (no kidney failure/eGFR<30) were chosen a priori. Still, our findings should be considered hypothesis-generating and warrant external

validation. Second, kidney function was determined by the creatinine-based CKD-EPI formula, without direct measurement of GFR. Although the CKD-EPI formula has displayed better performance than the Modification of Diet in Renal Disease (MDRD) equation [17], it is still possible that a few patients are misclassified. Third, VH-IVUS imaging was limited to a pre-specified target segment of a non-culprit coronary artery. This study design was chosen based on the hypothesis that such a non-stenotic segment reflects coronary wall pathophysiology of the larger coronary tree [34,35]. This hypothesis, on its part, was based on ex-vivo, as well as in-vivo studies using IVUS in patients with myocardial infarction. These studies have demonstrated the presence of TCFAs in places other than the culprit lesion or even culprit artery [16,36]. In fact, we were subsequently able to confirm this hypothesis, by demonstrating that imaging characteristics of the non-culprit artery are associated with increased risk of MACE within the current study population [34]. Therefore, this study design allows us to investigate whether the patient's burden and vulnerability of atherosclerotic disease e as reflected by the phenotype of a non-culprit artery segment e is associated with blood biomarkers [16]. Finally, although the spatial resolution of IVUS-VH is formally too low to detect thin caps, we have demonstrated that VH-IVUS derived TCFA lesions strongly and independently predict the occurrence of MACE within the current study population [34].

In conclusion, this study provides new insights into the role of plasma CysC and NGAL in coronary atherosclerosis. Most importantly, it shows that in patients with normal kidney function, those with higher CysC levels have fewer high-risk coronary lesions, while in patients with impaired kidneys, those with higher CysC have higher risk of MACE. Thus, this study implies that mild-to-moderate kidney dysfunction modifies the relationship between plasma CysC and coronary artery disease. This has not been established before, and it offers an explanation for the difference in findings observed in experimental and epidemiologic studies. With regard to cardiovascular risk prediction, CysC showed predictive capacities when $eGFR_{Cr}$ was below 90 mL/min/1.73m^2, whereas NGAL levels were not predictive of MACE.

References

1. Redon J. et al. Kidney function and cardiovascular disease in the hypertensive population: the ERIC-HTA study. J Hypertens 2006; 24: 663-669.
2. Shlipak M.G. et al. Cystatin C and prognosis for cardiovascular and kidney outcomes in elderly persons without chronic kidney disease. Ann Intern Med 2006; 145: 237-246.
3. C. Chronic Kidney Disease Prognosis, Matsushita K. et al. Association of estimated glomerular filtration rate and albuminuria with all-cause and cardiovascular mortality in general population cohorts: a collaborative meta-analysis. Lancet 2010; 375: 2073-2081.
4. Arbel Y. et al. Impact of estimated glomerular filtration rate on vascular disease extent and adverse cardiovascular events in patients without chronic kidney disease. Can J Cardiol 2013; 29: 1374-1381.
5. Shlipak M.G. et al. Update on cystatin C: new insights into the importance of mild kidney dysfunction. Curr Opin Nephrol Hy 2006; 15: 270-275.
6. Sukhova G.K. et al. Cystatin C deficiency increases elastic lamina degradation and aortic dilatation in apolipoprotein E-null mice. Circ Res 2005; 96: 368-375.
7. Shi G.P. et al. Cystatin C deficiency in human atherosclerosis and aortic aneurysms. J Clin Investig 1999; 104: 1191-1197.
8. Keppler D. Towards novel anti-cancer strategies based on cystatin function. Cancer Lett 2006; 235: 159-176.
9. Devarajan P. NGAL in acute kidney injury: from serendipity to utility. Am J Kidney Dis 2008; 52: 395-399.
10. Schmidt-Ott K.M. et al. Neutrophil gelatinaseassociated lipocalin-mediated iron traffic in kidney epithelia, Curr Opin Nephrol Hy 2006; 15: 442-449.
11. Hemdahl A.L. et al. Expression of neutrophil gelatinase-associated lipocalin in atherosclerosis and myocardial infarction. Arter Throm Vasc 2006; 26: 136-142.
12. Yan L. et al. The high molecular weight urinary matrix metalloproteinase (MMP) activity is a complex of gelatinase B/MMP-9 and neutrophil gelatinase-associated lipocalin (NGAL) - modulation of MMP-9 activity by NGAL. J Biol Chem 2001; 276: 37258-37265.
13. Galis Z.S. and Khatri J.J. Matrix metalloproteinases in vascular remodeling and atherogenesis - the good, the bad, and the ugly. Circ Res 2002; 90: 251-262.
14. Daniels L.B. et al. Plasma neutrophil gelatinaseassociated lipocalin is independently associated with cardiovascular disease and mortality in community-dwelling older adults the rancho bernardo study. J Am Coll Cardiol 2012; 59: 1101-1109.
15. Cheng J.M. et al. Circulating osteoglycin and NGAL/MMP9 complex concentrations predict 1-Year major adverse cardiovascular events after coronary angiography, Arter Thromb Vasc 2014; 34: 1078-1084.
16. de Boer S.P.M. et al. Relation of genetic profile and novel circulating biomarkers with coronary plaque phenotype as determined by intravascular ultrasound: rationale and design of the ATHEROREMO-IVUS study. Eurointervention 2014; 10: 953-960.
17. Levey A.S. and Stevens L.A. Estimating GFR using the CKD Epidemiology collaboration (CKD-EPI) creatinine equation: more accurate GFR estimates, lower CKD prevalence estimates, and better risk predictions. Am J Kidney Dis 2010; 55: 622-627.
18. F. National Kidney, K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Am J Kidney Dis 2002; 39: S1-S266.
19. Garcia-Garcia H.M. et al. Imaging of coronary atherosclerosis: intravascular ultrasound. Eur Heart J 2010; 31: 2456-2469C.

20. Nair A. et al. Automated coronary plaque characterization with intravascular ultrasound backscatter: ex vivo validation. Eurointervention 2007; 3: 113-120.
21. Rodriguez-Granillo G.A. et al. In vivo intravascular ultrasound-derived thin-cap fibroatheroma detection using ultrasound radiofrequency data analysis. J Am Coll Cardiol 2005; 46: 2038-2042.
22. Task Force on the management of, STseamiotESoC Steg P.G. et al. ESC Guidelines for the management of acute myocardial infarction in patients presenting with ST-segment elevation. Eur Heart J 2012; 33: 2569-2619.
23. Hamm C.W. et al. ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: the Task Force for the management of acute coronary syndromes (ACS) in patients presenting without persistent ST-segment elevation of the European Society of Cardiology (ESC). Eur Heart J 2011; 32: 2999-3054.
24. Schisterman E.F. et al. Optimal cut-point and its corresponding Youden Index to discriminate individuals using pooled blood samples. Epidemiology 2005; 16: 73-81.
25. Taglieri N. et al. Cystatin C and cardiovascular risk. Clin Chem 2009; 55: 1932-1943.
26. Bengtsson E. et al. Lack of the cysteine protease inhibitor cystatin C promotes atherosclerosis in apolipoprotein E-deficient mice. Arter Thromb Vasc 2005; 25: 2151-2156.
27. Galkina E. and Ley K. Immune and inflammatory mechanisms of atherosclerosis (*). Annu Rev Immunol 2009; 27: 165-197.
28. Xu Y.K. et al. Developmental regulation of synthesis and dimerization of the amyloidogenic protease inhibitor cystatin C in the hematopoietic system*. J Biol Chem 2014; 289: 9730-9740.
29. Zalba G. et al. Oxidative stress and atherosclerosis in early chronic kidney disease. Nephrol Dial Transpl 2006; 21: 2686-2690.
30. Janowski R. et al. Human cystatin C, an amyloidogenic protein, dimerizes through three-dimensional domain swapping. Nat Struct Biol 2001; 8: 316-320.
31. Lindberg S. et al. Prognostic utility of neutrophil gelatinase-associated lipocalin in predicting mortality and cardiovascular events in patients with ST-segment elevation myocardial infarction treated with primary percutaneous coronary intervention. J Am Coll Cardiol 2012; 60: 339-345.
32. Cruz D.N. et al. Neutrophil gelatinase-associated lipocalin as a biomarker of cardiovascular disease: a systematic review. Clin Chem Lab Med 2012; 50: 1533-1545.
33. Sahinarslan A. et al. Plasma neutrophil gelatinase-associated lipocalin levels in acute myocardial infarction and stable coronary artery disease. Coron Artery Dis 2011; 22: 333-338.
34. Cheng J.M. et al. In vivo detection of high-risk coronary plaques by radiofrequency intravascular ultrasound and cardiovascular outcome: results of the ATHEROREMO-IVUS study, Eur Heart J 2014; 35: 639-647.
35. Nicholls S.J. et al. Intravascular ultrasound-derived measures of coronary atherosclerotic plaque burden and clinical outcome. J Am Coll Cardiol 2010; 55: 2399-2407.
36. Libby P. Atherosclerosis: disease biology affecting the coronary vasculature. Am J Cardiol 2006; 98: 3Q-9Q.

Supplementary files

Supplementary Table 1. Plasma cystatin C, NGAL and presence of thin-cap fibroatheroma (VH-TCFA) lesions, lesions with plaque burden (PB)≥70%, and lesions with minimal luminal area (MLA) ≤4 mm².

	Unadjusted model	P	Multivariable model[c]	P
	OR (95% CI)		OR (95% CI)	
Total population				
VH-TCFA				
Cystatin C[a]	0.93 (0.78-1.10)	0.38	0.89 (0.74-1.08)	0.25
NGAL[b]	0.92 (0.76-1.10)	0.36	0.88 (0.72-1.07)	0.19
Plaque burden ≥70%				
Cystatin C[a]	0.93 (0.76-1.14)	0.50	0.75 (0.59-0.95)	0.018
NGAL[b]	0.95 (0.74-1.21)	0.67	0.92 (0.71-1.19)	0.51
MLA ≤4mm²				
Cystatin C[a]	0.90 (0.75 - 1.08)	0.27	0.79 (0.65-0.98)	0.028
NGAL[b]	1.00 (0.82 - 1.21)	0.95	1.01 (0.82-1.24)	0.90
ACS patients				
VH-TCFA				
Cystatin C[a]	0.86 (0.69-1.07)	0.18	0.80 (0.62-1.03)	0.085
NGAL[b]	0.86 (0.67-1.09)	0.21	0.85 (0.66-1.08)	0.18
Plaque burden ≥70%				
Cystatin C[a]	0.81 (0.61-1.09)	0.17	0.57 (0.40-0.81)	0.002
NGAL[b]	0.87 (0.62-1.24)	0.45	0.81 (0.56-1.17)	0.26
MLA ≤4mm²				
Cystatin C[a]	1.04 (0.79-1.28)	0.97	0.86 (0.65-1.14)	0.30
NGAL[b]	1.11 (0.85-1.44)	0.44	1.10 (0.84-1.44)	0.50
SAP patients				
VH-TCFA				
Cystatin C[a]	1.05 (0.81-1.37)	0.70	1.06 (0.79-1.42)	0.70
NGAL[b]	0.96 (0.70-1.30)	0.78	0.95 (0.69-1.31)	0.75
Plaque burden ≥70%				
Cystatin C[a]	1.04 (0.78-1.40)	0.77	1.00 (0.72-1.38)	0.97
NGAL[b]	1.01 (0.77-1.58)	0.60	1.05 (0.73-1.52)	0.79
MLA ≤4mm²				
Cystatin C[a]	0.77 (0.58-1.02)	0.071	0.73 (0.53-0.99)	0.044
NGAL[b]	0.91 (0.67-1.24)	0.55	0.90 (0.65-1.24)	0.51

ACS=acute coronary syndrome; SAP=stable angina pectoris. Multivariable model: adjusted for age, gender, diabetes mellitus, hypertension, indications for angiography, C-reactive protein. [a]Odds ratio (OR) per standard deviation increase ln-transformed cystatin C with 95% confidence interval (CI). [b]Odds ratio (OR) per standard deviation increase in NGAL with 95% confidence interval (CI).

Supplementary Table 2. Plasma cystatin C, NGAL and segment plaque volume, burden and VH-tissue types as determined by VH-IVUS, in the total population and stratified by indication for angiography.

	Cystatin C[a]	P	NGAL[b]	P
	β (95% CI)		β (95% CI)	
Total population				
Plaque burden[c]	0.03 (-0.05 – 0.11)	0.44	-0.02 (-0.11 – 0.07)	0.67
Plaque volume[c]	0.04 (-0.31 – 0.39)	0.83	-0.05 (-0.44 – 0.34)	0.79
Fibrous, %	-0.46 (-1.42 – 0.50)	0.35	0.52 (-0.54 – 1.58)	0.33
Fibro-fatty, %[c]	0.01 (-0.07 – 0.09)	0.86	0.05 (-0.04 – 0.13)	0.29
Necrotic core, %	-0.17 (-0.83 – 0.50)	0.62	-0.56 (-1.29 – 0.17)	0.13
Dense calcium, %[c]	0.09 (-0.01 – 1.19)	0.072	-0.07 (-0.18 – 0.04)	0.22
ACS patients				
Plaque burden[a]	0.01 (-0.10 – 0.12)	0.89	-0.05 (-0.16 – 0.07)	0.43
Plaque volume[a]	-0.22 (-0.68 – 0.24)	0.35	-0.27 (-0.77 – 0.23)	0.29
Fibrous, %	-0.20 (-1.50 – 1.10)	0.76	0.54 (-0.87 – 1.95)	0.45
Fibro-fatty, %[a]	-0.05 (-0.16 – 0.06)	0.39	0.08 (-0.04 – 0.20)	0.18
Necrotic core, %	-0.19 (-1.12 – 0.74)	0.69	-0.98 (-1.98 – 0.02)	0.055
Dense calcium, %[a]	0.12 (-0.01 – 0.24)	0.079	-0.06 (-0.20 – 0.08)	0.39
SAP patients				
Plaque burden[c]	0.05 (-0.07 – 0.16)	0.41	0.07 (-0.06 – 0.21)	0.29
Plaque volume[c]	0.35 (-0.20 – 0.90)	0.21	0.39 (-0.25 – 1.02)	0.23
Fibrous, %	-0.66 (-2.09 – 0.76)	0.36	0.03 (-1.61 – 1.67)	0.97
Fibro-fatty, %[c]	0.71 (-0.04 – 0.18)	0.22	0.03 (-0.10 – 0.16)	0.64
Necrotic core, %	-0.08 (-1.03 – 0.86)	0.86	-0.09 (-1.18 – 1.00)	0.87
Dense calcium, %[c]	0.04 (-0.12 – 0.19)	0.64	-0.02 (-0.20 – 0.16)	0.85

ACS=acute coronary syndrome; SAP=stable angina pectoris. [a]Unadjusted β per standard deviation increase in ln-transformed cystatin C with 95% confidence interval (CI). [b]Unadjusted β per standard deviation increase in NGAL with 95% confidence interval (CI). [c]Square root transformed.

Supplementary Table 3. Plasma cystatin C, NGAL and composite endpoint of all-cause mortality/acute coronary syndrome (ACS) in the total population and stratified by kidney function ($eGFR_{Cr}$).

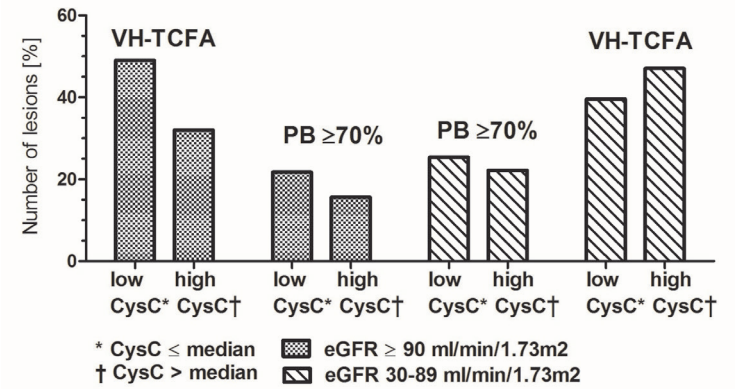
All-cause mortality / ACS	Unadjusted	P	Model 1	P	Model 2	P
	HR (95% CI)		HR (95% CI)		HR (95% CI)	
Total population						
Cystatin C[a]	1.67 (1.24 - 2.27)	<0.001	1.51 (1.08 - 2.10)	0.015	1.24 (0.88 - 1.77)	0.19
NGAL[b]	1.20 (0.85 - 1.71)	0.30	1.17 (0.81 - 1.70)	0.40	1.11 (0.77 - 1.61)	0.56
$eGFR_{Cr}$ ≥ 90 mL/min/1.73 m²						
Cystatin C[a]	1.39 (0.72 - 2.65)	0.32	1.33 (0.72 - 2.48)	0.36	1.11 (0.54 - 2.25)	0.78
NGAL[b]	1.04 (0.57 - 1.89)	0.89	1.12 (0.63 - 1.98)	0.69	1.08 (0.56 - 2.11)	0.81
$eGFR_{Cr}$ 30-89 mL/min/1.73 m²						
Cystatin C[a]	1.81 (1.23 - 2.66)	0.003	1.73 (1.17 - 2.55)	0.006	1.59 (1.01 - 2.50)	0.04
NGAL[b]	1.26 (0.80 - 1.98)	0.31	1.22 (0.77 - 1.94)	0.39	1.18 (0.74 - 1.87)	0.48

Model 1: adjusted for the age, gender, indication for angiography; Model 2: model 1 + diabetes mellitus, hypertension, C-reactive protein. Multivariable adjustment was constrained by the limited number of clinical endpoints. [a]Hazard ratio (HR) per standard deviation increase in ln-transformed cystatin C with 95% confidence interval (CI). [b]Hazard ratio (HR) per standard deviation increase in NGAL with 95% confidence interval (CI).

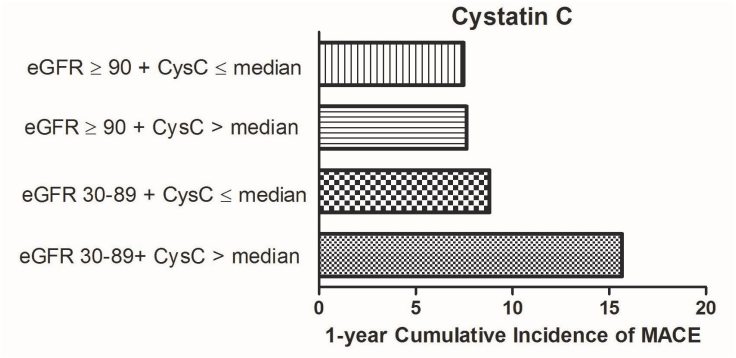
Supplementary Table 4. Plasma cystatin C, NGAL and major adverse coronary events (MACE) and the composite of all-cause mortality / acute coronary syndrome (ACS), stratified by indication for angiography.

	Unadjusted	P	Model 1	P	Model 2	P
	HR (95% CI)		HR (95% CI)		HR (95% CI)	
ACS patients						
MACE						
Cystatin C[a]	1.41 (1.01 - 1.98)	0.047	1.24 (0.85 - 1.81)	0.27	1.10 (0.73 - 1.65)	0.66
NGAL[b]	1.17 (0.80 - 1.70)	0.43	1.17 (0.80 - 1.72)	0.42	1.14 (0.76 - 1.69)	0.53
All-cause mortality / ACS						
Cystatin C[a]	1.61 (1.15 - 2.40)	0.007	1.47 (0.98 - 2.20)	0.06	1.23 (0.79 - 1.91)	0.37
NGAL[b]	1.33 (0.89 - 1.98)	0.16	1.35 (0.89 - 2.03)	0.15	1.33 (0.86 - 2.06)	0.20
SAP patients						
MACE						
Cystatin C[a]	1.39 (0.98 - 1.97)	0.07	1.35 (0.92 - 1.98)	0.12	1.25 (0.85 - 1.84)	0.26
NGAL[b]	1.18 (0.77 - 1.80)	0.44	1.10 (0.72 - 1.69)	0.66	1.08 (0.70 - 1.64)	0.73
All-cause mortality / ACS						
Cystatin C[a]	1.71 (1.02 - 2.88)	0.042	1.61 (0.90 - 2.87)	0.11	1.40 (0.76 - 2.59)	0.28
NGAL[b]	0.84 (0.42 - 1.70)	0.64	0.79 (0.39 - 1.61)	0.52	0.78 (0.38 - 1.58)	0.49

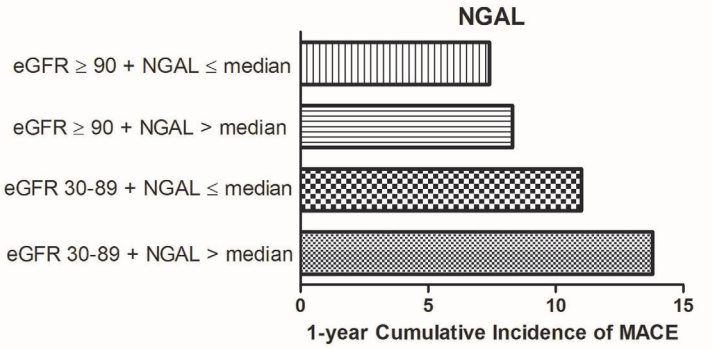
ACS=acute coronary syndrome; SAP=stable angina pectoris. Model 1: adjusted for the age, gender; Model 2: model 1 + diabetes mellitus, hypertension, C-reactive protein. [a]Hazard ratio (HR) per standard deviation increase in ln-transformed cystatin C with 95% confidence interval (CI). [b]Hazard ratio (HR) per standard deviation increase in NGAL with 95% confidence interval (CI).



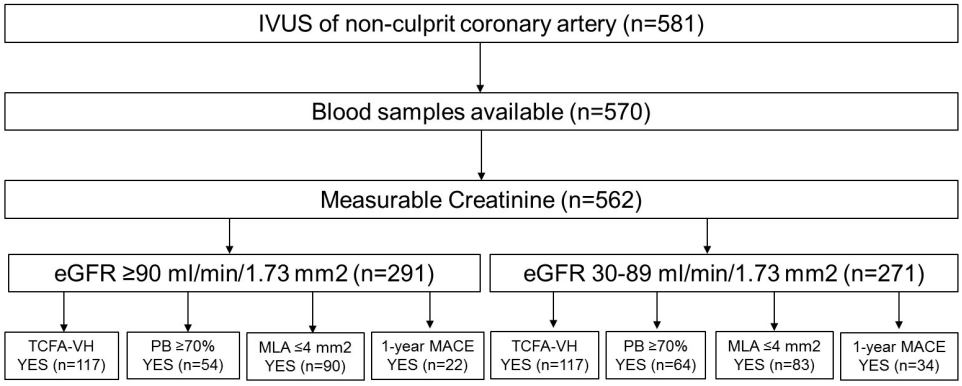
Supplementary Figure 1. Relative number of thin-cap fibroatheroma (VH-TCFA) lesions and lesions with plaque burden (PB)≥70% per strata of kidney function ($eGFR_{Cr}$) and plasma cystatin C (CysC) levels above and below median.



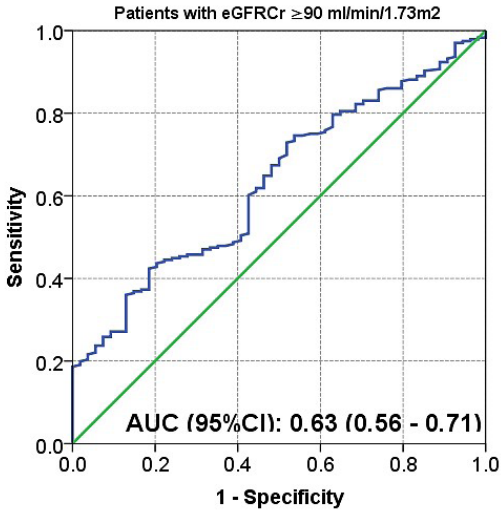
Supplementary Figure 2A. Cumulative incidence (1-year) of major adverse coronary events (MACE) per strata of kidney function (eGFRCr) and plasma cystatin C (CysC) levels above and below median.



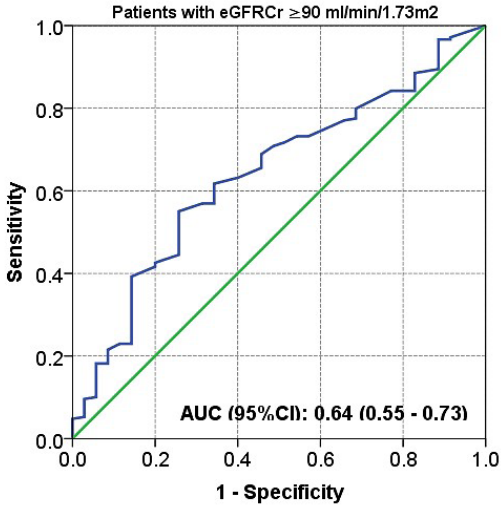
Supplementary Figure 2B. Cumulative incidence (1-year) of major adverse coronary events (MACE) per strata of kidney function ($eGFR_{Cr}$) and plasma NGAL levels above and below median.



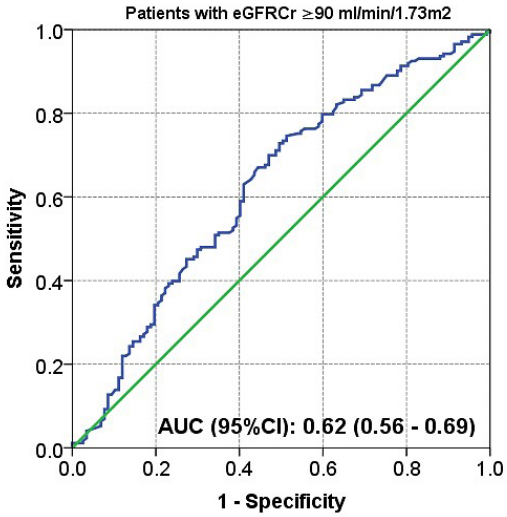
Supplementary Figure 3. Absolute numbers of thin-cap fibroatheroma (VH-TCFA) lesions, lesions with plaque burden (PB) ≥70%, lesions with minimal luminal area (MLA) ≤4 mm^2 and 1-year major adverse coronary events (MACE) per strata of kidney function ($eGFR_{Cr}$).



Supplementary Figure 4. Receiver operator characteristic (ROC) curve of plasma cystatin C (CysC) for the prediction of absence of lesion with plaque burden (PB) ≥70% in patients with $eGFR_{Cr}$ ≥90 ml/min/1.73m².
CysC of 773.0 ng/ml is optimal cut-off value, based on Youden index (highest sum of sensitivity and specificity -1), discriminating between patients who did not have lesion with PB ≥70% (CysC ≥773.0 ng/ml), and those who had (CysC <773.0 ng/ml). AUC (95%CI), area under the ROC curve with corresponding 95% confidence interval.

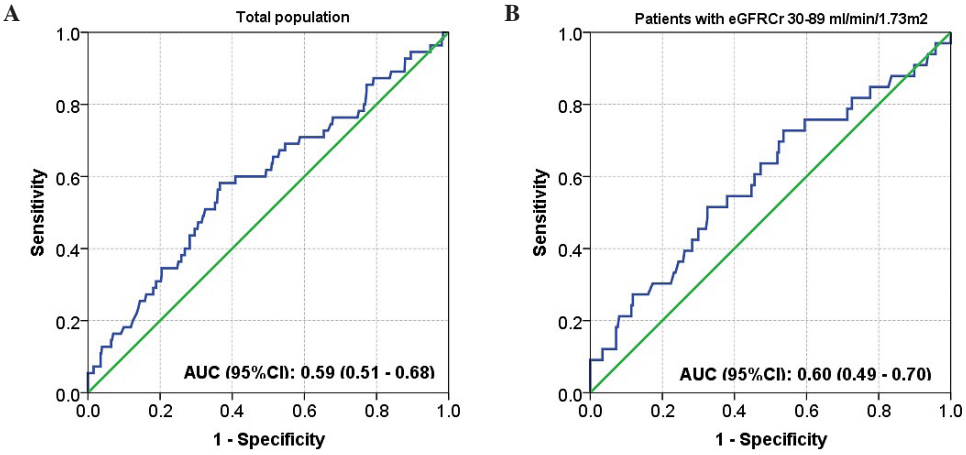


Supplementary Figure 5. Receiver operator characteristic (ROC) curve of plasma NGAL for the prediction of absence of lesion with plaque burden (PB)≥70% in patients with $eGFR_{Cr}$ ≥90 ml/min/1.73m².
NGAL of 180.0 ng/ml is optimal cut-off value, based on Youden index (highest sum of sensitivity and specificity -1), discriminating between patients who did not have lesion with PB≥70% (NGAL ≥180.0 ng/ml) and those who had (NGAL <180.0 ng/ml). AUC (95%CI), area under the ROC curve with corresponding 95% confidence interval.



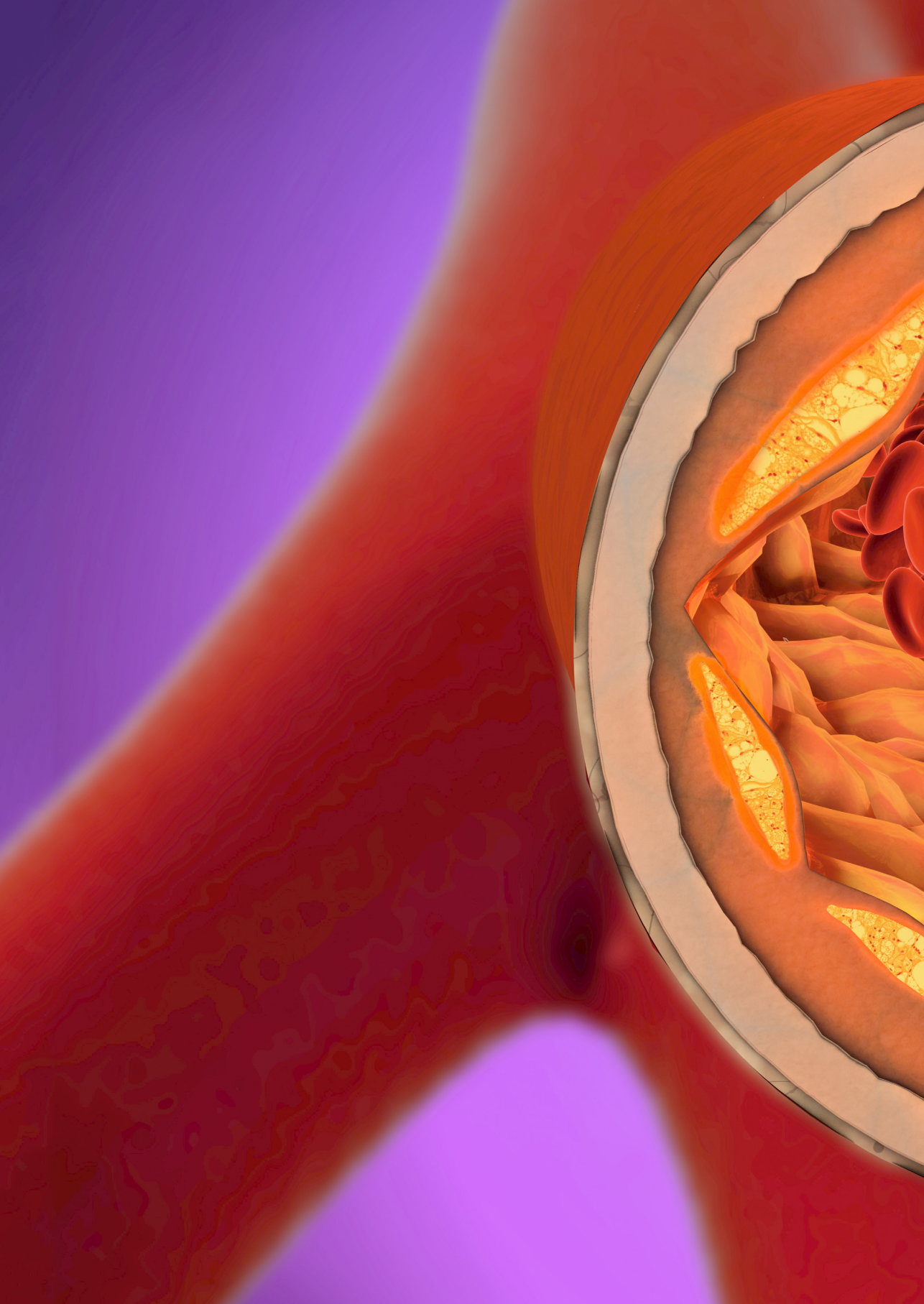
Supplementary Figure 6. Receiver operator characteristic (ROC) curve of plasma cystatin C (CysC) for the prediction of absence of thin-cap fibroatheroma (VH-TCFA) lesion in patients with $eGFR_{Cr}$ ≥90 ml/min/1.73m².

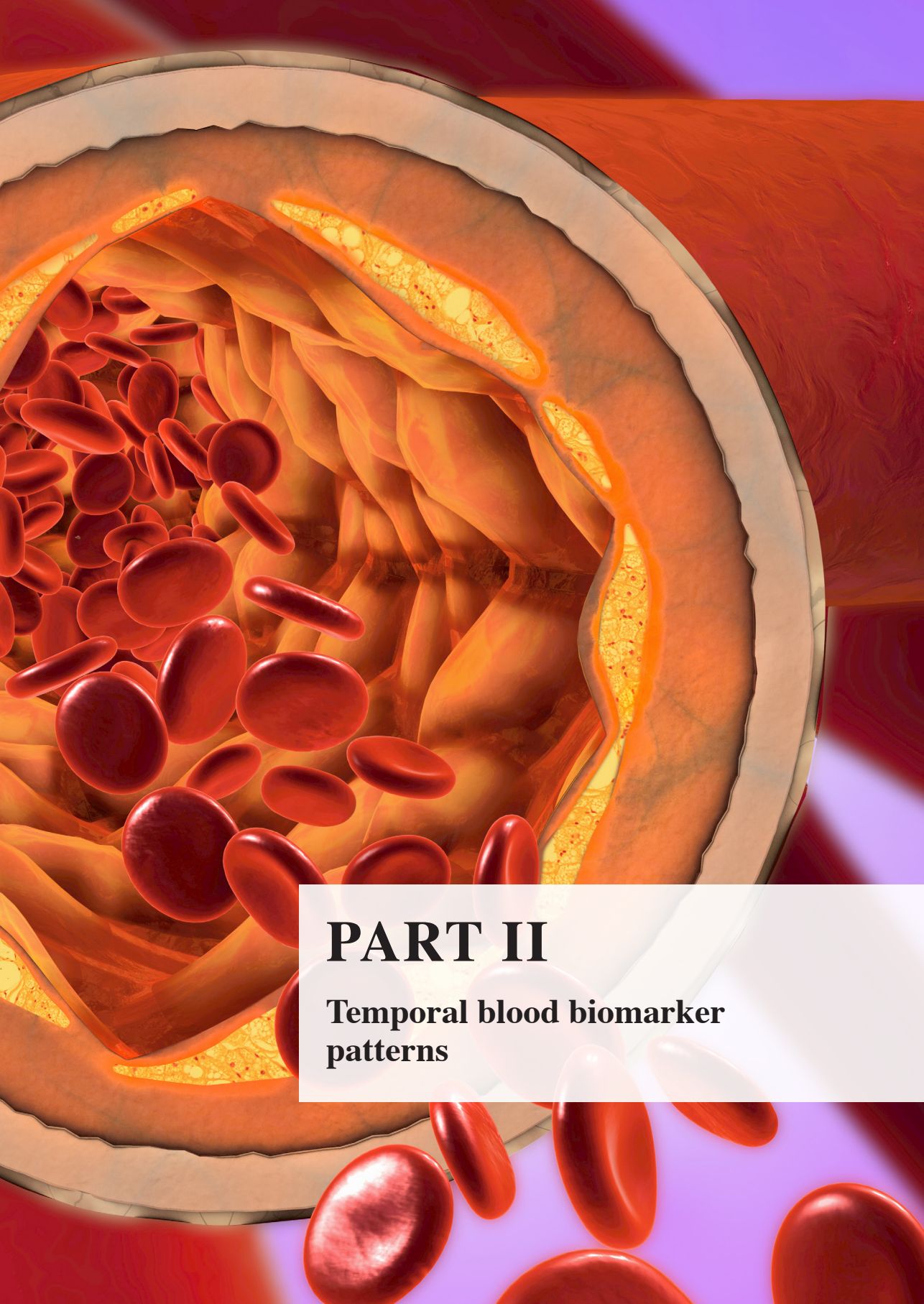
CysC of 678.5 ng/ml is optimal cut-off value, based on Youden index (highest sum of sensitivity and specificity -1), discriminating between patients who did not have VH-TCFA lesion (CysC ≥678.5 ng/ml), and those who had (CysC <678.5 ng/ml). AUC (95%CI), area under the ROC curve with corresponding 95% confidence interval.



Supplementary Figure 7. Receiver operator characteristic (ROC) curve for plasma cystatin C (CysC) for the prediction of the occurrence of major adverse coronary events (MACE) in total population and in patients with $eGFR_{Cr}$ 30-89 ml/min/1.73m².

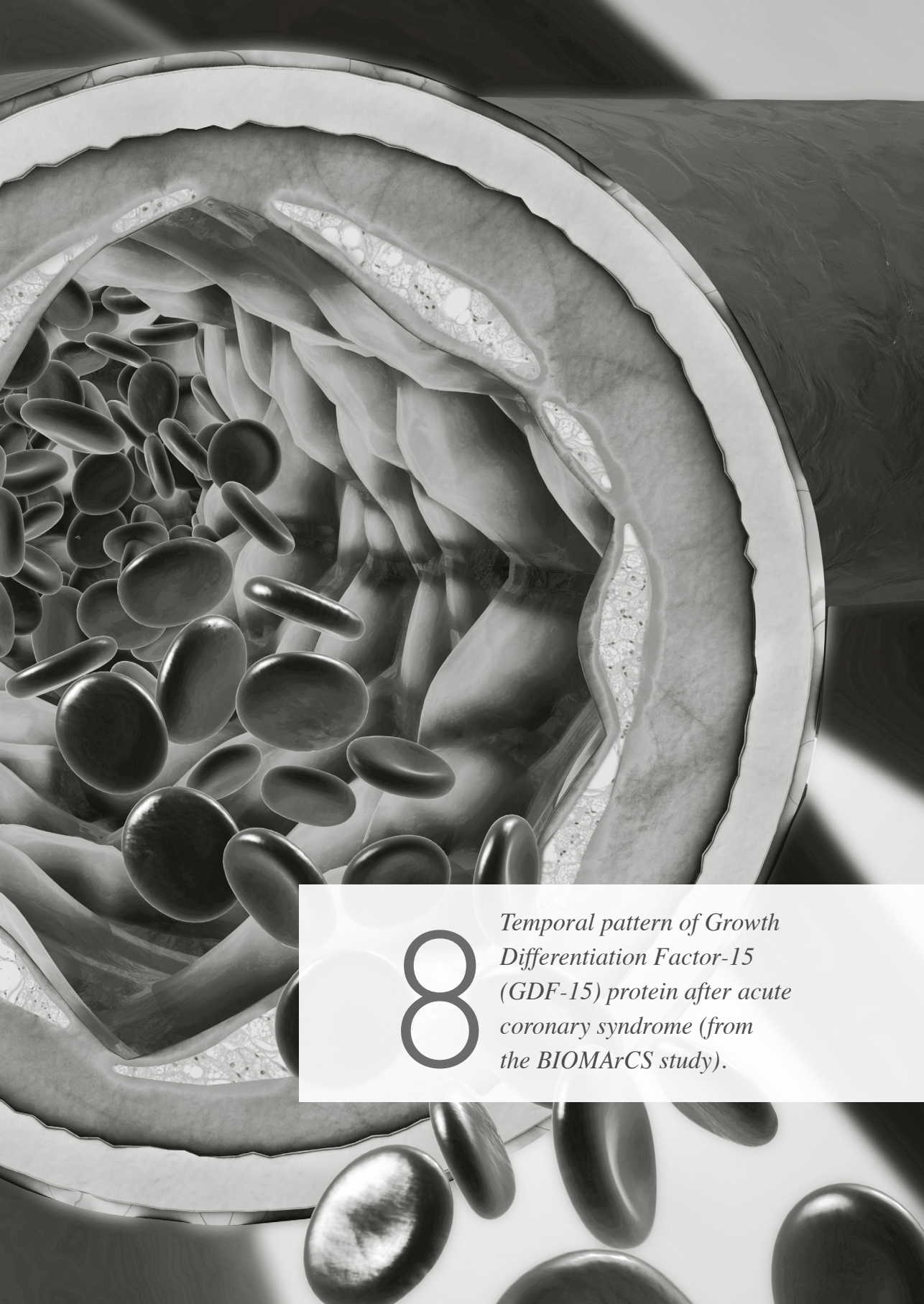
CysC of 849.0 ng/ml is optimal cut-off value, based on Youden index (highest sum of sensitivity and specificity -1), discriminating between patients who developed MACE (CysC ≥849.0 ng/ml) and those who did not (CysC <849.0 ng/ml). AUC (95% CI), area under the ROC curve with corresponding 95% confidence interval.





PART II

Temporal blood biomarker patterns



8

Temporal pattern of Growth Differentiation Factor-15 (GDF-15) protein after acute coronary syndrome (from the BIOMArCS study).

8

Temporal pattern of Growth Differentiation Factor-15 (GDF-15) protein after acute coronary syndrome (from the BIOMArCS study).

Buljubasic N, Vroegindewey MM, Oemrawsingh RM, Asselbergs FW, Cramer E, Liem A, van der Harst P, Maas A, Ronner E, Schotborgh C, Wardeh AJ, Akkerhuis KM, Boersma E.

Am J Cardiol 2019; 124(1): 8-13. doi: 10.1016/j.amjcard.2019.03.049.

Abstract

Rationale: Growth differentiation factor-15 (GDF-15) has appeared as a promising biomarker with strong predictive abilities in acute coronary syndrome (ACS). However, studies are solely based on *single* measurements in the *acute* phase of an ACS event. The way GDF-15 patterns in post ACS patients behave on the long term is largely unknown.

Methods & Results: We conducted a nested case-control study within our multicenter, prospective, observational biomarker study (BIOMArCS) of 844 ACS patients. Following an index ACS event, high-frequency blood sampling was performed during 1-year of follow-up. GDF-15 was determined batchwise by electrochemiluminescence immunoessays in 37 cases with a recurrent event during 1-year follow-up, and in 74 event-free controls. Cases and controls had a mean ± standard deviation age of 66.9 ± 11.3 years and 81% were men. From 30 days onwards, patients showed stable levels, which were on average 333 (95% confidence interval 68-647) pg/mL higher in cases than controls (1704 vs. 1371 pg/mL; p-value 0.013). Additionally, in the post 30-day period, GDF-15 showed low within-individual variability in

both cases and controls.

Conclusions: In conclusion, post ACS patients experiencing a recurrent event had stable and systematically higher GDF-15 levels during 30-day to 1-year follow-up than their event-free counterparts with otherwise similar clinical characteristics. Thus, post-discharge blood sampling might be used throughout the course of 1 year to improve prognostication, whereas, in view of the low within-individual variation, the number of repeated sampling moments might be limited.

Introduction

In recent years, circulating growth differentiation factor-15 (GDF-15), a stress-induced cytokine, has emerged as a biomarker of interest due to its potential prognostic value in patients with cardiovascular disease [1]. In particular, elevated levels of GDF-15 are associated with an impaired prognosis after acute coronary syndromes (ACS) [2-6]. However, the prognostic value of GDF-15 in ACS patients thus far, has been mainly based on *single* measurements in the *early*, *acute* phase of an acute ischemic event. Therefore, the optimal time point in the stabilized post ACS phase for GDF-15 blood sampling to make prognostic implications remains not fully elucidated yet. We used our 'BIOMarker study to identify the Acute risk of a Coronary Syndrome' (BIOMArCS) with high-frequency blood sampling in post ACS patients as a platform to describe the temporal evolution of GDF-15 during 1-year follow-up, to evaluate differences between patients with and without a recurrent event, and to study the individual variability of GDF-15.

Methods

We performed a nested case-control analysis within the main BIOMArCS study that was approved by the medical ethics committees of all participating hospitals. The rationale and design of BIOMArCS are described in detail elsewhere [7]. In brief, BIOMArCS is a prospective, multicenter, observational study conducted in 18 participating hospitals in the Netherlands. A total of 844 patients, admitted for an ACS, including unstable angina pectoris, non-ST-

elevation myocardial infarction (NSTEMI) and ST-elevation myocardial infarction (STEMI) and with at least one additional cardiovascular risk factor, were enrolled between 2008 and 2015. Patients underwent regular blood sampling after the initial admission for ACS according to a strict schedule to describe the temporal evolution of blood biomarkers in the post ACS phase, and to reveal deviations in temporal biomarker patterns prior to a recurrent coronary event. Venipuncture was performed at admission, at hospital discharge and subsequently every fortnight during the first half year, followed by monthly blood sample collection until 1 year. Follow-up blood sampling was terminated permanently after coronary artery bypass grafting, hospital admission for heart failure, or a detoriation of renal function leading to a glomerular filtration rate of < 30 mL/min/1.73m^2, since these conditions influence circulating biomarker concentrations. Ultimately, patients had 17 (median) repeated blood samples within 1 year. The study was performed in accordance with the criteria described in the declaration of Helsinki and all patients provided written informed consent for their participation.

The primary study endpoint was a composite of cardiac death, non-fatal myocardial infarction, or unstable angina pectoris, requiring urgent coronary revascularization within 1-year follow-up. Study endpoints were adjudicated by a clinical event committee, blinded for any biomarker data, after the study was completed in 2015. In 2014, Roche Diagnostics GmbH offered the opportunity to determine GDF-15 with their pre-commercial assay in a limited number of BIOMArCS patients. Since no commercial GDF-15 assay would have been available within the foreseeable future, we decided to accept this one-time offer. We analyzed the blood samples of all patients with an investigator-reported endpoint event at that time, as well as 2 matched endpoint-free controls for each such event. Matching was based on admission hospital, age (± 5 year range), gender, diabetes mellitus, peripheral artery disease and history of coronary artery disease (CAD). We kept the results unanalyzed until study completion and event-adjudication. After study completion, it appeared that 37 of the investigator-reported events were confirmed as study endpoint. In the current analysis, these events were included as cases, together with their corresponding 74 matched controls.

Blood samples were initially handled and securely stored on-site. Aliquots were frozen at -80 degrees Celsius within two hours after withdrawal. Long-term storage and batchwise GDF-15 analysis took place at the department of Clinical Chemistry of the Erasmus MC, Rotterdam (the Netherlands). Laboratory personnel were blinded for any clinical data, including endpoint data. The plasma GDF-15 concentrations were measured using the quantitative sandwich electrochemiluminescence immunoassay "ECLIA" (Roche Diagnostics, Mannheim, Germany)

on a Cobas e601 immunoassay analyzer. The lowest detection limit of GDF-15 analyte concentration was 400 pg/mL. No interference was found using in vitro tests to determine interference between 51 commonly used cardiovascular pharmaceuticals and the assay.

It is important to discern a fixed amount of individual biomarker variability from clinically relevant changes over time. Therefore, certain parameters have been described to define individual variability, which are needed to interpret the relevance of changes of repeated measurements. The coefficient of variation (CV) of a series of measurements is defined as 100% times the standard deviation (sd) of the measurements divided by their mean value ($\overline{X}$):

$$CV = 100\% * sd/\overline{X}$$

According to the methods by Fraser and Harris [8], the total variation of a series of repeated measurements in individual subjects can be split in 3 components, which represent the variation due to the imprecision of the analytical process (CV_a), the intra-individual or within-subject variation (CV_i), and the inter-individual or between-subject variation (CV_g). CV_a of GDF-15 in our laboratory appeared to be 1.75% and 1.88% for high and low concentrations, respectively. Subsequently, CV_i was defined as the median value of the CVs of the repeated measurements in individual subjects ($CV_{subject}$), adjusted for the analytical variation:

$$CV_i = \sqrt{\text{median}(CV_{subject}^2) - CV_a^2}$$

Finally, CV_g was determined as 100% times the standard deviation ($sd_{\overline{X}_{subject}}$) of the mean values of the repeated measurements in individual subjects ($\overline{X}_{subject}$) by the (unweighted) mean of these means ($\overline{X}_{group}$):

$$CV_g = 100\% * sd_{\overline{X}_{subject}}/\overline{X}_{group}$$

The Index of Individuality (II) is the ratio of the combined within-subject and analytical variation relative to the between-subject variation:

$$II = \sqrt{CV_i^2 + CV_a^2}/CV_g$$

Since a high II (>1.4) indicates a relatively high within-subject variation and low between-subject variation, it is more likely that an unusual biomarker value will lie outside the borders of most overlapping values and therefore population-based reference intervals are sufficient. Conversely, when the II is low (<0.6), it is agreed that subjects should have their own reference values, based on previous samples. The Reference Change Value (RCV) reflects the limit of

(relative) change in biomarker values in individual subjects that can be explained by the combined within-subject and analytical variation. For biomarkers with a skewed distribution a log-normal approach has been described, and the RCV limits can be determined as follows:

$$RCV_{downward} = e^{-Z_{\alpha/2}*\sqrt{2\ln(CV_i^2+CV_a^2+1)}} - 1$$

$$RCV_{upward} = e^{Z_{\alpha/2}*\sqrt{2\ln(CV_i^2+CV_a^2+1)}} - 1$$

We used $\alpha = 0.05$ (for 95% confidence), thus $Z_{0.025} = 1.96$.

Since GDF-15 is known to be initially elevated post ACS, all aforementioned variability parameters are based on >30 day blood samples. Although the exact pathophysiological substrate for an initial elevation of GDF-15 in the acute phase of ACS is unknown [9], a possible 'washout' effect due to an acute phase reaction is thereby hampered. After that period, biochemical as well as clinical stabilization are expected to be reached for adequately determining GDF-15 variability. Thereafter, only patients with at least three available measurements in that time window are included, leaving 20 cases and 46 controls for variability analysis.

Categorical data are presented as numbers and percentages. Continuous variables are presented as mean±standard deviation (SD) or as median and interquartile range (IQR), depending on their distribution. Normality of continuous variables was examined by visual inspection of the histogram and by normal Q-Q plots. The examined biomarker GDF-15 (outcome) showed a skewed distribution and was therefore ^{2}log-transformed for further analyses. GDF-15 biomarker trajectories were examined across different follow-up time intervals after the ACS index event during one year of follow-up. Within the first 7 days from admission, each patient's maximum biomarker value was determined. The median values of the patient-level maximum were compared between the cases and controls by linear mixed effect (LME) models. Then, the patient-average biomarker trajectories between 7 and 30 days from admission and from 30 days onwards were compared between cases and controls by LME models with nested random effects. Time from index ACS event until each blood sample measurement and a group variable (case / control) were entered as fixed effects in the model, paired individuals as random effects and serial GDF-15 measurements as the dependent variable (model 1). Subsequently, we fitted multivariable LME models with adjustment for age and gender (model 2), and with additional adjustment for admission diagnosis, diabetes mellitus, smoking, hypertension, hypercholesterolemia, BMI, history of revascularization, history of myocardial infarction and

serum creatinine value (which was measured at each sampling moment) (model 3). Values were eventually backtransformed to present mean differences (95% confidence intervals (CI)) between cases and controls on the linear scale. All data were analyzed with SPSS (version 21) and R statistical software (version 3.5.1). All statistical tests were two-tailed and p-values <0.050 were considered statistically significant.

Results

Baseline clinical characteristics are presented in Table 1.

Table 1. Baseline clinical characteristics (n = 111).

Variable	Cases (n = 37)	Controls (n = 74)	P-value
Age (years)	67.9±11.7	66.3±11.2	0.79
Men	30 (81%)	60 (81%)	0.95
ST-segment elevation myocardial infarction	13 (35%)	42 (57%)	<0.001
Non-ST-segment elevation myocardial infarction	18 (49%)	27 (37%)	0.17
Unstable angina pectoris	6 (16%)	5 (7%)	0.78
Smoker			
Current	13 (35%)	25 (34%)	0.91
Former	11 (30%)	23 (31%)	0.97
Never	13 (35%)	26 (35%)	0.95
Diabetes mellitus	12 (32%)	26 (35%)	0.52
Hypertension	19 (51%)	40 (54%)	0.77
Hypercholesterolemia	17 (46%)	30 (41%)	0.81
Prior myocardial infarction	12 (32%)	23 (30%)	0.93
Prior percutaneous coronary intervention	11 (30%)	20 (27%)	0.88
Prior coronary artery bypass grafting	9 (24%)	10 (14%)	0.64
Prior stroke	8 (22%)	7 (10%)	0.78
Prior peripheral vascular disease	10 (27%)	13 (18%)	0.73

Values are mean±standard deviation or n (%). P-values were obtained by the linear mixed model (continuous variable) or generalized linear mixed model (categorical variable), whichever was appropriate.

The matching procedure appeared successful, as there were no relevant differences between cases and controls, except for admission diagnosis of STEMI (p-value <0.001). During the first 7 days after the index ACS, GDF-15 levels reached maximum values (median [IQR]) of 2436 [2286 – 4236] pg/mL in cases and 1804 [1207 – 3749] pg/mL in the controls (p-value 0.22). These levels slightly decreased within the first 30 days, and the mean value within the 7 to 30 day period was 1908 pg/mL and 1590 pg/mL in cases and controls, respectively. This

mean difference of 318 (95% CI ranging from -215 to 1058) pg/mL was statistically non-significant (p-value 0.26). From 30 days after the index ACS onwards until 1-year follow-up, cases had systematically higher GDF-15 levels than controls (Table 2, Figure 1). This difference remained significant after correction for age, gender and multiple cardiovascular risk factors (p-value 0.013). These findings are confirmed in strata according to gender, diabetes mellitus, smoking, serum creatinine value and admission diagnosis (Supplementary Tables 1 - 5). No differences were observed in GDF-15 levels across the various subgroups (all p-values for heterogeneity were >0.05).

Table 2. Mean GDF-15 (pg/mL) values in cases and controls in the 30 days to 1 year period after ACS admission.

	Cases	Controls	Mean difference (95% CI)	P-value
Model 1[a]	1780	1414	366 (26 - 788)	0.034
Model 2[b]	1744	1415	329 (2 - 732)	0.049
Model 3[c]	1704	1371	333 (68 - 647)	0.013

CI=confidence interval. [a]Unadjusted for patient characteristics. [b]Adjusted for age and gender. [c]Adjusted for age, gender, admission diagnosis, diabetes mellitus, smoking, hypertension, hypercholesterolemia, BMI, history of revascularization, history of myocardial infarction and serum creatinine value (measured at each time-point).

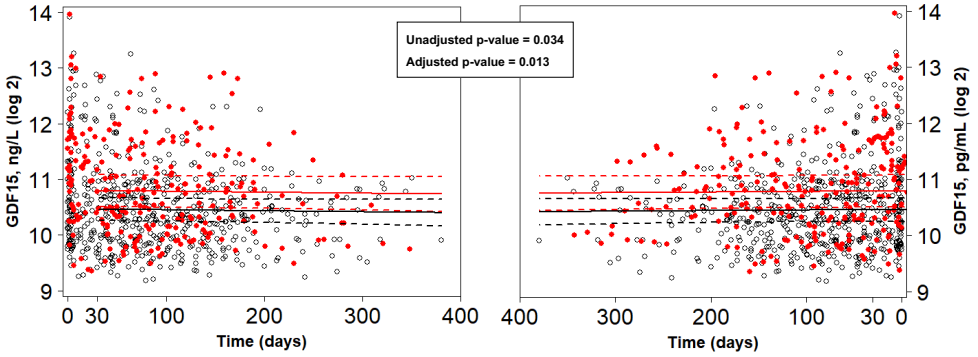


Figure 1. Serial measurements and temporal evolvement of GDF-15 (pg/mL) in cases (red) and controls (black). The left graph shows the evolvement of GDF-15 since the index event (t=0) until 1-year follow-up. The right graph shows the evolvement of GDF-15 before the study endpoint (t=0 in cases), or until the last blood sample moment (t=0 in controls). The points represent measurements in individual patients. The lines represent the group average values (bold lines) and the 95% confidence intervals (dashed lines), using linear mixed models with nested random effects.

An overview of the different variability parameters, calculated for a selected amount of cases and controls, is presented in Table 3. With a CV_a of 2%, both groups displayed limited within-subject variability (CV_i of 16.3 for the cases and 11.5 for the controls), whereas the

between-subject variability showed larger variation (CV_g of 73.1 for the cases and 62.0 for the controls). This is also shown by a plot (Figure 2), which illustrates low within-subject variability (CV_i / (CV_i + CV_g) = 16-18%) and large between-subject variability (CV_g / (CV_i + CV_g) 82-84%) with a minimum of 579 pg/mL and a maximum of 9748 pg/mL. As could be expected from low within-subject variability and high between-subject variability in both groups, the II was low (below the threshold value of 0.6), and thus individual reference values are preferred. Thereby we found that the limits of change between subsequent measurements (RCV) are allowed to range from -36% to 57% in cases and from -28% to 38% in controls.

Table 3. Parameters describing the biological variability of GDF-15 serial measurements 30 days after the acute coronary syndrome index event in both cases and controls.

	Cases (n = 20)	Controls (n = 46)
Average biomarker level (pg/mL), median [IQR]	1423 [1122 - 2594]	1317 [966 - 1705]
Analytical coefficient of variation (CV_a)	2%	2%
Intra-individual coefficient of variation (CV_i)	16.3	11.5
Inter-individual coefficient of variation (CV_g)	73.1	62.0
Index of individuality (II)	0.2	0.2
Reference change value (RCV)	45%	32%
Reference change value, upper limit	57%	38%
Reference change value, lower limit	-36%	-28%

Parameters describing the biological variability of GDF-15, as calculated by formulas presented in the method section.

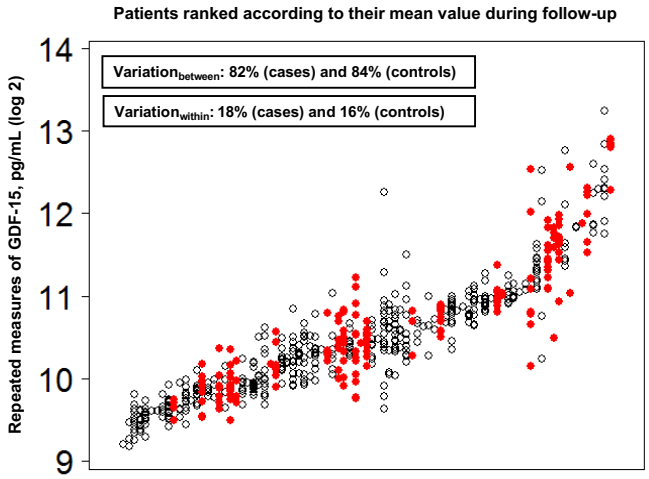


Figure 2. Graphical illustration of GDF-15 variability by displaying the distribution of GDF-15 measurements per patient 30 days after the ACS index event.

The data points represent measurements in individual patients (cases in red; controls in black), ranked according to their mean value during post 30 days follow-up in order to display within- and between-individual variation.

Discussion

This is the first study to describe GDF-15 patterns in post ACS patients in great detail, utilizing a high-frequency blood sampling design during 1 year. Four key lessons were learned from our analysis. First, in individual patients, after reaching a peak value in the first week after admission, GDF-15 concentrations levelled off to levels that remain stable throughout 1-year follow-up. Second, importantly, there was no steady or sudden change in GDF-15 level prior to a recurrent event. Thus, no significant changes in GDF-15 values occurred after the initial post ACS phase. Third, patients who experienced a recurrent event had on average 26% higher GDF-15 levels than those who remained event-free. Although the prognostic value of GDF-15 has already been demonstrated by previous studies with one baseline measurement, we additionally proved that repeated post-discharge blood sampling of GDF-15 during 1 year might help improve accurate prognostication. Fourth, within-patient variability was much smaller than between-patient variability, meaning that the number of repeated blood samples to obtain a patient-specific stable GDF-15 level can be limited.

Considering the natural course of GDF-15 post ACS in our analysis, peak values are present in the first 7 days after an ACS, whereafter it seems that GDF-15 subtly reaches a stabilized phase without significant changes, especially prior to a recurrent event. By the use of frequent serial measurements, the stability of the marker in individual patients on the long term was established. This finding is supported by previous data in post ACS patients, demonstrating that GDF-15 concentrations show small alterations through the first 72 hours of hospitalization and potentially several months thereafter [3,4]. The fact that GDF-15 concentration levels remain significantly higher in patients who experience a recurrent event than in event-free patients over the course of a year in our study without any level changes around the event, suggests that GDF-15 is not merely a reflection of extent of myocardial damage or infarct size, but rather reflects severity of (chronic) atherosclerotic disease burden at any time point. This proposition is further supported by findings with cardiovascular magnetic resonance, demonstrating that GDF-15 is unrelated to infarct size and myocardial area at risk 2-4 days after the index event [9]. Furthermore, GDF-15 concentrations on admission seemed to be similar between NSTEMI and STEMI patients, of whom more severe myocardial damage can be expected [2,3]. Thus, in support of our hypothesis, previous studies do not indicate that GDF-15 solely mirrors tissue damage.

With regard to prognostication, GDF-15 has been thoroughly investigated in clinical

studies and shown to be an independent prognostic marker of mortality and cardiovascular events in both healthy individuals and CAD patients, which is in accordance with our results [4-6, 9-11]. Specifically, a recent meta-analysis focused on ACS patients, including 8 studies and 8903 participants, showed a significant hazard ratio (95% confidence interval) of 1.66 (1.47 – 1.87) on the association between GDF-15 and mortality or recurrent MI [6]. However, most studies performed blood sampling only on admission at the onset of an ACS or at discharge during the recovery phase of an ACS. As we have demonstrated, initial GDF-15 peak values were largely present in the first 7 days after the index ACS, which is likely the expression of an acute phase reaction. Therefore, single blood samples timed in the early phase during the course of an ACS event may represent a peak level, which does not clarify its prognostic implications on long term post ACS. To our knowledge, only two clinical studies have performed a limited number of serial GDF-15 measurements in post NSTEMI patients [3,4]. Wollert et al [3] collected blood samples on admission and at 24, 48 and 72 hours in a subgroup of 399 patients, whereas Eggers et al [4] measured GDF-15 at baseline and after clinical stabilization at 6 weeks, 3 months, 6 months in 950 patients. Both studies found significant associations with respectively 1-year and 5-year mortality at each time point. Along with our data with highly frequent blood sampling, we have additionally demonstrated that obtained blood samples within a course of 1 year post ACS will provide comparable prognostic information.

The biological variability of GDF-15 in ACS patients has not been described so far. We found low within-subject variability and high between-subject variability, which corresponds with findings from a study on the biovariability of GDF-15 conducted in 41 patients with stable chronic systolic dysfunction [12]. In this study, GDF-15 was measured at four blood sampling time points up until 3 months and showed very little biological (within-)variation, while there was an elevated between-individual variation (reflected by a low II). Altogether, describing biomarker variability is warranted to provide insight into the significance and interpretation of a biomarker in clinical practice. Our results indicate that changes in serial measurements of GDF-15 in an individual who experienced an ACS, independently of disease status (case or control), might be more useful than population derived reference values.

The unique design and character of this study enabled us to provide novel data on the temporal evolution and variability of GDF-15 post ACS. Nevertheless, some limitations warrant to be acknowledged. Due to the study design and its observational character, this substudy is unable to demonstrate causal inference. Whether GDF-15 merely reflects CAD

pathways, or directly contributes to coronary pathophysiology remains unknown. Also, as opposed to previous studies with large cohorts, we could not demonstrate significant differences in GDF-15 levels between cases and controls within the first 30 days. This is probably due to a lack of power with a limited number of measurements <30 days within a relatively small cohort. In line with this, we are aware of the fact that our study comprises a relatively small number of study patients and events. Further, by acknowledging previous studies that investigated the prognostic value of GDF-15 in large study populations, our study encompassing an exceptional blood sampling frequency method should rather be seen as hypothesis-testing with an extension to existing knowledge.

In conclusion, with detailed analysis of the longitudinal GDF-15 pattern post ACS, we have demonstrated that GDF-15 concentrations remain stable during follow-up with limited within-individual variation. In patients who eventually experience a recurrent event, GDF-15 is systematically elevated, independently of clinical risk factors and serum creatinine. Thus, to enable risk stratification with GDF-15 in post ACS patients, blood sampling might be used throughout the course of 1 year for prognostication, whereas the number of repeated sampling moments might be limited. Further exploration of the exact role of GDF-15 in risk stratifying post ACS patients and deciding on clear cut off points is warranted in future studies in order to make accurate prognostications.

References

1. Wollert K.C. et al. Growth Differentiation Factor 15 as a Biomarker in Cardiovascular Disease. Clin Chem 2017; 63: 140-151.
2. Kempf T. et al. Growth-differentiation factor-15 improves risk stratification in ST-segment elevation myocardial infarction. Eur Heart J 2007; 28: 2858-2865.
3. Wollert K.C. et al. Prognostic value of growth-differentiation factor-15 in patients with non-ST-elevation acute coronary syndrome. Circulation 2007; 115: 962-971.
4. Eggers K.M. et al. Growth-differentiation factor-15 for long-term risk prediction in patients stabilized after an episode of non-ST-segment-elevation acute coronary syndrome. Circ Cardiovasc Genet 2010; 3: 88-96.
5. Khan S.Q. et al. Growth differentiation factor-15 as a prognostic marker in patients with acute myocardial infarction. Eur Heart J 2009; 30: 1057-1065.
6. Zhang S. et al. Growth differentiation factor-15 predicts the prognoses of patients with acute coronary syndrome: a meta-analysis. BMC Cardiovasc Disord 2016; 16: 82.
7. Oemrawsingh R.M. et al. Cohort profile of BIOMArCS: the BIOMarker study to identify the Acute risk of a Coronary Syndrome-a prospective multicentre biomarker study conducted in the Netherlands. BMJ Open 2016; 6: e012929.
8. Fraser C.G. and Harris E.K. Generation and application of data on biological variation in clinical chemistry. Crit Rev Clin Lab Sci 1989; 27: 409-437.
9. Eitel I. et al. Growth-differentiation factor 15 as predictor of mortality in acute reperfused ST-elevation myocardial infarction: insights from cardiovascular magnetic resonance. Heart 2011; 97: 632-640.
10. Rohatgi A. et al. Association of growth differentiation factor-15 with coronary atherosclerosis and mortality in a young, multiethnic population: observations from the Dallas Heart Study. Clin Chem 2012; 58: 172-182.
11. Kempf T. et al. Growth-differentiation factor-15 for risk stratification in patients with stable and unstable coronary heart disease: results from the AtheroGene study. Circ Cardiovasc Genet 2009; 2: 286-292.
12. Frankenstein L. et al. Reference change values and determinants of variability of NT-proANP and GDF15 in stable chronic heart failure. Basic Res Cardiol 2009; 104: 731-738.

Supplementary files

Supplementary Table 1. GDF-15 (pg/mL) values of cases and controls in strata according to gender in the 30 days to 1 year period after index ACS.

	Men			Women			P for heterogeneity
	Cases	Controls	Mean difference (95% CI)	Cases	Controls	Mean difference (95% CI)	
Model 1[a]	1822	1446	377 (-10 - 867)	1512	1185	326 (-251 - 1262)	0.97
Model 2[b]	1806	1452	354 (-15 - 817)	1505	1227	279 (-281 - 1170)	0.96
Model 3[c]	1773	1329	444 (142 - 809)	1506	1619	-113 (-596 - 598)	0.10

CI=confidence interval. Mean GDF-15 values were based on nested linear mixed effects models with (^{2}log-transformed) GDF-15 as the dependent variable, and with gender, time from index ACS event to the blood sample measurement and group variable (case / control) as the main independent variables. To obtain the p-value for heterogeneity, an interaction term (group variable * gender) was added to the model. [a]Unadjusted for patient characteristics. [b]Adjusted for age. [c]Adjusted for age, admission diagnosis, diabetes mellitus, smoking, hypertension, hypercholesterolemia, body mass index, history of revascularization, history of myocardial infarct and serum creatinine value (measured at each time-point).

Supplementary Table 2. GDF-15 (pg/mL) values of cases and controls in strata according to diabetes mellitus in the 30 days to 1 year period after index ACS.

	Diabetes			Non-diabetes			P for heterogeneity
	Cases	Controls	Mean difference (95% CI)	Cases	Controls	Mean difference (95% CI)	
Model 1[a]	2131	1816	315 (-324 - 1228)	1605	1208	397 (37 - 860)	0.57
Model 2[b]	2038	1772	266 (-324 - 1096)	1607	1236	371 (22 - 815)	0.56
Model 3[c]	2175	1760	415 (-169 - 1213)	1508	1215	293 (9 - 643)	0.98

CI=confidence interval. Mean GDF-15 values were based on nested linear mixed effects models with (^{2}log-transformed) GDF-15 as the dependent variable, and with presence of diabetes mellitus, time from index ACS event to the blood sample measurement and group variable (case / control) as the independent variables. To obtain the p-value for heterogeneity, an interaction term (group variable * diabetes mellitus) was added to the model. [a]Unadjusted for patient characteristics. [b]Adjusted for age and gender. [c]Adjusted for age, gender, admission diagnosis, smoking, hypertension, hypercholesterolemia, body mass index, history of revascularization, history of myocardial infarct and serum creatinine value (measured at each time-point).

Supplementary Table 3. GDF-15 (pg/mL) values of cases and controls in strata according to smoking in the 30 days to 1 year period after index ACS.

	Smoking			Non-smoking			P for heterogeneity
	Cases	Controls	Mean difference (95% CI)	Cases	Controls	Mean difference (95% CI)	
Model 1[a]	1410	1265	145 (-294 - 784)	1997	1458	539 (65 - 1158)	0.38
Model 2[b]	1518	1392	126 (-326 - 771)	1889	1411	478 (50 - 1411)	0.36
Model 3[c]	1832	1552	280 (-226 - 978)	1646	1317	330 (-31 - 791)	0.78

CI=confidence interval. Mean GDF-15 values were based on nested linear mixed effects models with (^{2}log-transformed) GDF-15 as the dependent variable, and with smoking status, time from index ACS event to the blood sample measurement and group variable (case / control) as the independent variables. To obtain the p-value for heterogeneity, an interaction term (group variable * smoking status) was added to the model. [a]Unadjusted for patient characteristics. [b]Adjusted for age and gender. [c]Adjusted for age, gender, admission diagnosis, diabetes mellitus, hypertension, hypercholesterolemia, body mass index, history of revascularization, history of myocardial infarct and serum creatinine value (measured at each time-point).

Supplementary Table 4. GDF-15 (pg/mL) values of cases and controls in strata according to creatinine levels (µmol/L) in the 30 days to 1 year period after index ACS.

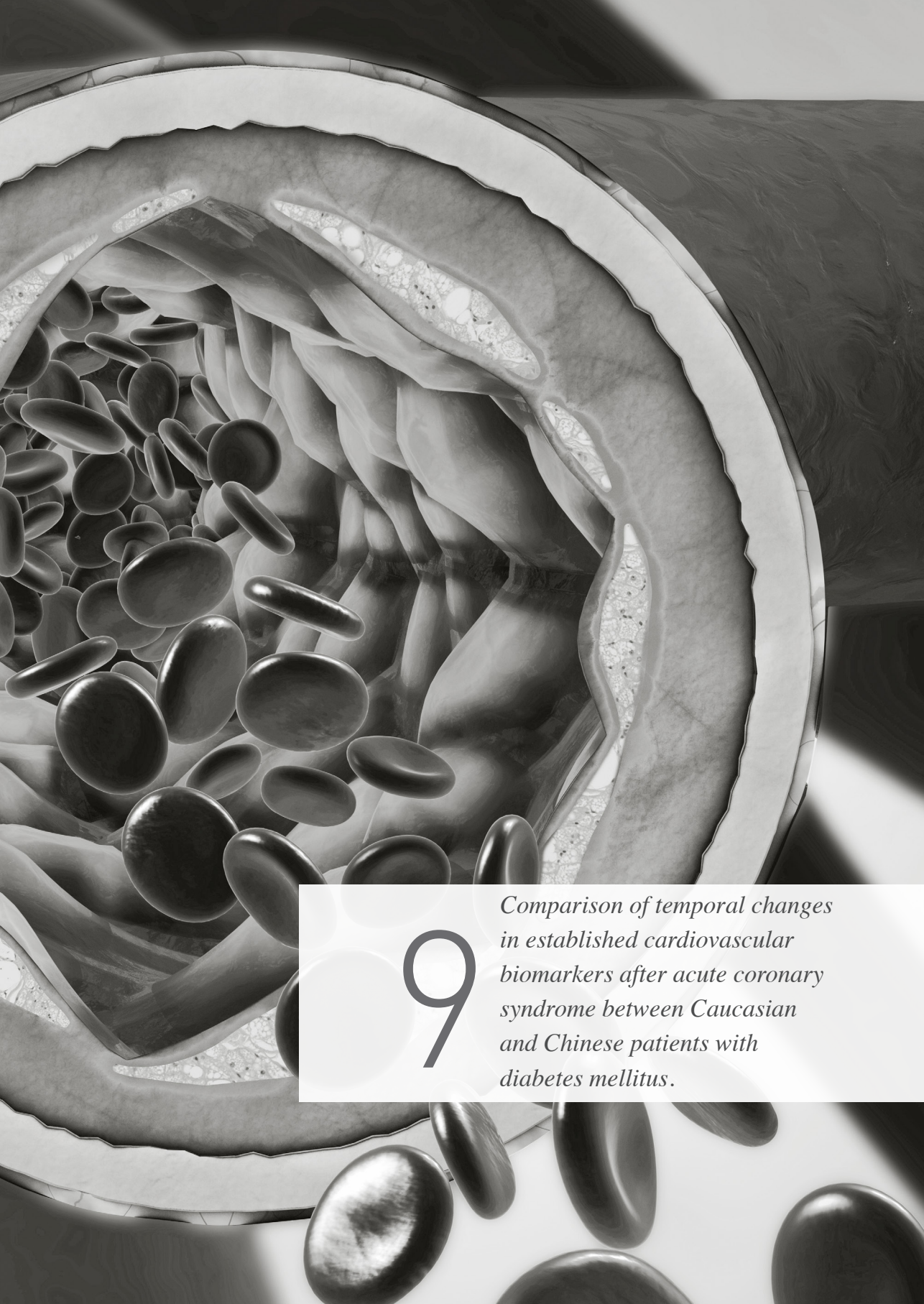
	Creatinine ≥ 85			Creatinine < 85			P for heterogeneity
	Cases	Controls	Mean difference (95% CI)	Cases	Controls	Mean difference (95% CI)	
Model 1[a]	1952	1532	420 (-79 - 1091)	1490	1288	203 (-220 - 794)	0.68
Model 2[b]	1640	1518	122 (-92 - 977)	1545	1319	226 (-193 - 800)	0.78
Model 3[c]	1817	1460	357 (-25 - 841)	1553	1336	217 (-151 - 698)	0.71

CI=confidence interval. Mean GDF-15 values were based on nested linear mixed effects models with (^{2}log-transformed) GDF-15 as the dependent variable, and with serum creatinine level (dichotomized into equal or above the median and below the median), time from index ACS event to the blood sample measurement and group variable (case / control) as the independent variables. To obtain the p-value for heterogeneity, an interaction term (group variable * (dichotomous) serum creatinine level) was added to the model. [a]Unadjusted for patient characteristics. [b]Adjusted for age and gender. [c]Adjusted for age, gender, admission diagnosis, smoking, diabetes mellitus, hypertension, hypercholesterolemia, body mass index, history of revascularization and history of myocardial infarction.

Supplementary Table 5. GDF-15 (pg/mL) values of cases and controls in strata according to admission diagnosis in the 30 days to 1 year period after index ACS.

	STEMI			NSTEMI			P for heterogeneity
	Cases	Controls	Mean difference (95% CI)	Cases	Controls	Mean difference (95% CI)	
Model 1[a]	1535	1223	312 (-119 - 911)	1758	1792	-34 (-502 - 604)	0.29
Model 2[b]	1573	1275	299 (-123 - 876)	1746	1725	20 (-428 - 624)	0.36
Model 3[c]	1670	1337	333 (-68 - 862)	1748	1485	264 (-140 - 789)	0.76

CI=confidence interval; STEMI=ST-elevation myocardial infarction; NSTEMI=non-ST-elevation myocardial infarction. Mean GDF-15 values were based on nested linear mixed effects models with (^{2}log-transformed) GDF-15 as the dependent variable, and with admission diagnosis, time from index ACS event to the blood sample measurement and group variable (case / control) as the independent variables. To obtain the p-value for heterogeneity, an interaction term (group variable * admission diagnosis) was added to the model. [a]Unadjusted for patient characteristics. [b]Adjusted for age and gender. [c] Adjusted for age, gender, smoking, diabetes mellitus, hypertension, hypercholesterolemia, body mass index, history of revascularization, history of myocardial infarct and serum creatinine value (measured at each time-point).



9

Comparison of temporal changes in established cardiovascular biomarkers after acute coronary syndrome between Caucasian and Chinese patients with diabetes mellitus.

9

Comparison of temporal changes in established cardiovascular biomarkers after acute coronary syndrome between Caucasian and Chinese patients with diabetes mellitus.

Buljubasic N, Zhao W, Cheng JM, Li H, Oemrawsingh RM, Akkerhuis KM, Yu H, Zhou L, Wu Y, Boersma E, Gao W.

Submitted.

Abstract

Rationale: Population means of conventional cardiovascular biomarkers are known to differ between ethnic groups. In this study we performed detailed comparisons in the temporal pattern of these biomarkers between Caucasian and Chinese diabetic patients with coronary artery disease.

Methods & Results: We studied differences in temporal changes of established cardiovascular biomarkers, including high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides, total cholesterol, cardiac Troponin T (TnT), NT-proBNP and C-reactive protein (CRP), in 48 Chinese and 48 matched Caucasian patients with type 2 diabetes mellitus who were admitted for ACS. Matching factors included admission diagnosis, gender, age, history of CAD, and cardiovascular risk factors. Blood samples were collected at regular time intervals during 30 days to 1 year after the index ACS. Altogether a median of 16 and 9 repeated samples per patient were available in Caucasians and Chinese, respectively. The biomarker trajectories were analyzed by linear mixed effect models with adjustments

for medication use. In the >30 day post ACS period, mean serum levels of LDL (2.16 vs 1.47 mmol/L; P-value <0.001), total cholesterol (4.08 vs 3.11 mmol/L; P-value <0.001), triglycerides (2.20 vs 1.08 mmol/L; P-value <0.001), TnT (11.0 vs. 7.76 ng/L; P-value 0.010) and CRP (2.0 vs 0.78 mg/L; P-value <0.001) were systematically higher in Caucasian than in Chinese patients. HDL and NT-proBNP levels were similar.

Conclusions: Our study showed clinically relevant differences in serum levels of established cardiovascular biomarkers between Caucasian and Chinese post ACS patients. Further cross-ethnic studies are warranted to determine secondary prevention treatment biomarker targets in specific populations.

Introduction

For decades coronary artery disease (CAD) has been the leading cause of mortality and morbidity worldwide [1, 2]. Global analyses have demonstrated a favourable trend in economically developed (Western) countries with declining (age-standardized) CAD mortality rates over the past decennia, whereas its incidence is increasing in non-Western regions [1, 3, 4]. Various factors are attributable for this epidemiological shift, but fact is that CAD has become a major burden to non-Western societies [5].

Recognition of CAD onset in the asymptomatic phase is the cornerstone of successful primary prevention. Also, in patients with established disease, the success of secondary prevention depends on early recognition of individuals with high risk of cardiovascular (CV) events. Blood biomarkers, reflecting underlying pathophysiological processes, can be instrumental in this respect [6]. For example, inflammatory markers, such as C-reactive protein (CRP) and interleukin-18, have been extensively studied and shown to be a valuable predictor for adverse outcomes in patients with CAD [7, 8]. Thus far, most CV biomarkers have been merely validated in Caucasians, and little is known about their generalizability to other ethnic groups. Furthermore, existing inter-ethnic CV biomarker studies have focused on general populations [9], and biomarker data in CAD populations from different ethnic groups are scarce. Finally, inter-ethnic biomarker studies are typically characterized by cross-sectional designs, with single measurements at a certain baseline moment. Hence, the observed results might easily be affected by accidental factors. Insight into longitudinal biomarker patterns by

means of repeated blood sampling may nullify these random variations, and thus reveal true differences in biomarker levels between populations.

The need for biomarker validation in order to optimize secondary prevention strategies is especially warranted in Asia, where CAD now is an upcoming epidemic [5]. Concerns especially exist in the (rural) Chinese population, where an increasing number of patients with coronary heart disease is leading to rapidly increasing mortality rates [10]. Similar to this worrisome trend are the rising numbers in prevalence of diabetes mellitus type 2 (DM2), which has become a serious health concern, leading to the world's largest epidemic in China [11, 12]. The joint effect of established coronary heart disease and prevalent diabetes markedly increase the risk of coronary mortality [13]. Biomarkers should especially be further investigated in these high risk groups, since they are particularly prone to recurrent events and might benefit most from secondary prevention strategies. Nevertheless, biomarker studies in Asian populations have been mainly focused on South Asians [9]. But, within Asia, there is broad geographical variation in patient risk profiles, which makes it unlikely that findings from South Asians can easily be extrapolated to Chinese individuals. In fact, far less biomarkers have been investigated in Chinese cohorts, residing in their country of origin.

Against this background, we evaluated differences in levels of serum high-density lipoprotein (HDL), low-density lipoprotein (LDL), total cholesterol, triglycerides, cardiac Troponin T (TnT), N-terminal fragment of pro-brain natriuretic peptide (NT-proBNP) and CRP - which we consider the most relevant CV biomarkers - between Dutch-Caucasian and Chinese DM2 patients presenting with acute coronary syndrome (ACS). In particular, we aimed to reveal inter-ethnic differences in the temporal evolution of these CV biomarkers during 1 year following the index ACS event.

Methods

Study design and patients

Figure 1 describes a patient flow diagram to illustrate the flow of participants through the study. We selected 48 Chinese ACS patients with established DM2 from the 'Peking and Rotterdam on Mission to Reduce Coronary Artery Disease' (PRoMISS) study. PRoMISS is a prospective, observational study, conducted in 12 hospitals in the larger area of Beijing (China), and enrolled patients during 2013 to 2014 admitted for an ACS and with a clinical

diagnosis of DM2 prior to this index event. The definition of ACS covered unstable angina pectoris (UAP), non-ST elevation myocardial infarction (NSTEMI) and ST-elevation myocardial infarction (STEMI). Blood samples (non-fasting) were taken from PRoMISS patients at the day of hospital admission, at the day of hospital discharge, followed by monthly blood sample collection until 1 year follow-up. Altogether a median of 9 repeated samples per patient were available in the Chinese cohort.

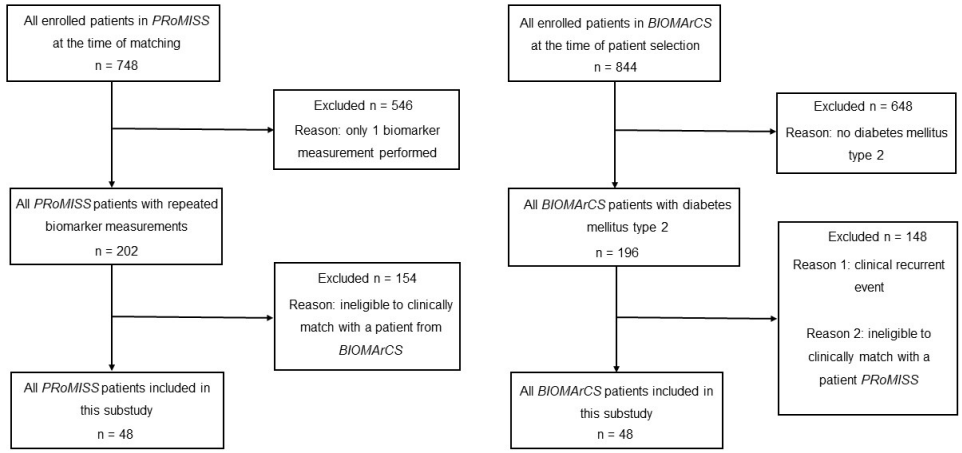


Figure 1. Study flow chart.

A patient flow diagram according to the international STARD guidelines to report flow of participants through the study.

Subsequently, we selected 48 DM2-ACS patients with Caucasian ethnicity from the Dutch prospective, observational 'BIOMarker study to identify the Acute risk of a Coronary Syndrome' (BIOMArCS) [14]. BIOMArCS enrolled 844 ACS patients with and without diabetes mellitus in 18 participating hospitals in The Netherlands during 2008 to 2015. A subgroup of 23% (n=196) in this cohort was diagnosed with DM2. Patients underwent blood sampling at admission, at the day of hospital discharge and subsequently every fortnight during the first 6 months after discharge, followed by monthly blood sample collection until 1 year. A median of 16 repeated samples per patient were available in BIOMArCS. Chinese-PRoMISS and Caucasian-BIOMArCS patients were 1:1 matched on age (± 5 year range), sex, admission diagnosis, history of CAD, and risk factor profile, including diabetes mellitus, hypercholesterolemia, hypertension, peripheral vascular disease and smoking. At the time

of matching, from the completed BIOMArCS study 48 diabetic patients could be optimally paired according to the matching criteria with 48 patients from the ongoing PRoMISS study. None of the patients experienced a recurrent ACS event throughout follow-up.

The PRoMISS and BIOMArCS studies were approved by the medical ethics committees of the participating hospitals. All participating patients from both studies provided written informed consent. Information on baseline characteristics and medication use was directly derived from patients' medical records and prospectively entered into a dedicated database.

Biomarker analysis

In PRoMISS and BIOMArCS, after preparation, aliquots were frozen at -80 degrees Celsius within two hours after withdrawal. Blood samples were initially handled and securely stored on-site, and then transported to the central laboratory of the Peking University Third Hospital, Beijing, China (PRoMISS) or the Erasmus MC, Rotterdam, the Netherlands (BIOMArCS) for long-term storage. After completion of data collection, blood samples were analysed batch-wise on the Roche Cobas® 8000 analysis platform, using the following reagens (Roche Diagnostics Mat.-No./ Genisys-No): HDL: 04399803190; LDL: 03038866322; total cholesterol: 03039773190; triglycerides: 20767107322; TnT: 05092728190; NT-proBNP: 05390109190; CRP: 04628918190. PRoMISS and BIOMArCS samples were analysed in two different central laboratories (in China and the Netherlands), but the reagens had the same production lot number and analysis protocols were identical.

Statistical analysis

Categorical data are presented as numbers and percentages. Continuous variables are presented as mean ± standard deviation (SD) in case of a normal distribution, or as median and interquartile range (IQR) in case of a skewed distribution. Normality of the distributions of continuous variables was examined by visual inspection of the histogram and by normal Q-Q plots. The measured biomarkers showed a skewed distribution and were therefore log2-transformed for analysis. Missing values on baseline characteristics and medication use were minimal (7% missing information on gender and 3% missings on type of medication used) and addressed by complete case analysis. There were no missing values on biomarker levels.

Differences in baseline clinical characteristics between the Chinese-PRoMISS and Caucasian-BIOMArCS patients were evaluated by the paired samples t-test (for continuous variables), McNemar test (for categorical variables) or Marginal Homogeneity (for categorical

variables with more than two categories) to account for the 1:1 matching.

Linear mixed effect (LME) models were applied with nested random effects (to account for the paired data). A grouping variable (PRoMISS or BIOMArCS) and time were entered as fixed effects, and paired individuals were entered as random effects, to determine mean biomarker levels in both cohorts. Interaction terms (grouping variable x time) were added (as fixed effects) to determine differences in biomarker evolution over time between the PRoMISS-Chinese and BIOMArCS-Caucasian patients. LME analyses were conducted with biomarker values on the log2 scale, but the main results are presented on the linear scale for ease of interpretation.

A substantial difference in medication use was observed between the PRoMISS-Chinese and BIOMArCS-Caucasian patients, which could have influenced biomarker levels. Therefore, adjusted LME models were constructed, including medication use (aspirin, statins, beta-blockers, ACE-inhibitors or angiotensin-II receptor blockers, nitrates and anti-diabetics) as potential confounding factors. Thereby a certain amount of potential bias was addressed.

Data analyses were performed with SPSS (version 21) and RStudio software (version 1.0.136). All statistical tests were two-tailed and p-values <0.050 were considered statistically significant.

Results

Patient characteristics

Baseline clinical characteristics of the two successfully matched patient study cohorts are presented in Table 1. The BIOMArCS-Caucasian and PRoMISS-Chinese patients had a mean age of 60.2 ± 8.0 years and 60.0 ± 8.1, respectively. Most patients were men (BIOMArCS 87.5%, PRoMISS 85.4%), presenting with a STEMI (64.4% in both cohorts). As expected after matching, baseline clinical characteristics and cardiovascular risk factors were similarly distributed in the two study cohorts. Overall, commonly prescribed cardiovascular drugs after an ACS were more frequently used in the BIOMArCS-Caucasian patients than in the PRoMISS-Chinese patients, in particular beta-blockers (BIOMArCS 91.7%, PRoMISS 68.8%, p=0.007), nitrates (BIOMArCS 25.0%, PRoMISS 0%, p=<0.001) and anti-diabetics (BIOMArCS 89.6%, PRoMISS 68.8% p=0.021).

Table 1. Baseline clinical characteristics of the (matched) study patients.

	BIOMArCS (n = 48)	PRoMISS (n = 48)	P-value
Patient characteristics			
Age, years	60.2 ± 8.0	60.0 ± 8.1	0.89
Male gender, n (%)	42 (87.5)	41 (85.4)	0.25
Admission diagnosis, n (%)			1.00
STEMI	31 (64.6)	31 (64.6)	
NSTEMI	10 (20.8)	10 (20.8)	
UAP	7 (14.6)	7 (14.6)	
Cardiovascular risk factors, n (%)			
Smoking			0.56
Current	20 (41.7)	16 (33.3)	
Former	11 (22.9)	14 (29.2)	
Never	17 (35.4)	18 (37.5)	
Diabetes Mellitus	48 (100.0)	48 (100.0)	1.00
Hypertension	31 (64.6)	33 (68.8)	0.80
Hypercholesterolemia	27 (56.2)	25 (52.1)	0.79
Medical history, n (%)			
Previous myocardial infarction	10 (20.8)	7 (14.6)	0.55
Previous PCI	10 (20.8)	7 (14.6)	0.51
Previous CABG	5 (10.4)	0 (0.0)	0.06
Previous stroke	2 (4.2)	2 (4.2)	1.00
History of peripheral vascular disease	2 (4.2)	0 (0.0)	0.50
Medication use at first blood sample moment >30 days after admission, n (%)			
Aspirin	46 (95.8)	42 (87.5)	0.29
Statin	45 (93.8)	37 (77.1)	0.06
Beta-blocker	44 (91.7)	33 (68.8)	0.007
ACE-inhibitor or ARB	38 (79.2)	29 (60.4)	0.06
Nitrates	12 (25.0)	0 (0.0)	<0.001
Anti-diabetics	43 (89.6)	33 (68.8)	0.021

ARB=angiotensin II receptor blocker; CABG=coronary artery bypass graft surgery; NSTEMI=non-ST-segment elevation myocardial infarction; STEMI=ST-segment elevation myocardial infarction; UAP=unstable angina pectoris. Values are expressed as mean ± standard deviation or proportion, n (%). P-values were obtained by paired samples t-test (for the continuous variable), McNemar test (for categorical variables) or Marginal Homogeneity (for categorical variables with more than two categories), whichever was appropriate.

Biomarker trajectories

BIOMArCS-Caucasian patients had statistically significant higher mean longitudinal levels for most lipid biomarkers than their PRoMISS-Chinese counterparts. Especially, clinically relevant higher mean LDL (2.16 vs 1.47 mmol/L; P-value <0.001), total cholesterol (4.08 vs 3.11 mmol/L; P-value <0.001) and triglycerides (2.20 vs 1.08 mmol/L) levels were found (Figure 2, Table 2). The estimated mean lipid biomarker levels did not change during the study period in BIOMArCS-Caucasian patients (Figure 2, Supplementary Table 1). In PRoMISS-

Chinese patients, however, these biomarkers had a slight, but statistically significant, tendency to increase over time. Since the monthly increase was only 0.5% (cholesterol) to 4.3% (HDL) of the longitudinal mean level, the differences between BIOMArCS and PRoMISS remained fairly constant during the >30 days post ACS study period.

With respect to the established non-lipid cardiovascular biomarkers that we studied: BIOMArCS-Caucasians had higher mean longitudinal levels of TnT (11.0 vs 7.76 ng/L; P-value 0.010) and CRP (2.07 vs 0.78 mg/L; P-value <0.001) than PRoMISS-Chinese patients (Figure 3, Table 2). NT-proBNP levels were similar. In both cohorts, TnT, NT-proBNP and CRP slightly decreased over time (Figure 3, Supplementary Table 1), with a somewhat steeper decline in NT-proBNP in PRoMISS. Again, however, the monthly changes were far smaller than the longitudinal mean levels, so that the BIOMArCS-PRoMISS differences in mean levels were factually time-independent.

Table 2. Mean biomarker levels in the study patients 30 days to 1 year after the index ACS admission.

	BIOMArCS (n = 48)	PRoMISS (n = 48)	Mean difference (95% CI)	P-value
HDL, mmol/L	0.97 (0.81 - 1.15)	0.94 (0.81 - 1.10)	0.03 (-0.06 - 0.13)	0.491
LDL, mmol/L	2.16 (1.60 - 2.94)	1.47 (1.11 - 1.89)	0.69 (0.40 - 1.04)	<0.001
Cholesterol, mmol/L	4.08 (3.34 - 4.92)	3.11 (2.66 - 3.64)	0.97 (0.62 - 1.37)	<0.001
Triglycerides, mmol/L	2.20 (1.51 - 3.24)	1.08 (0.80 - 1.44)	1.12 (0.78 - 1.52)	<0.001
Troponin T, ng/L	11.04 (6.40 - 18.10)	7.76 (5.07 - 12.27)	3.28 (0.71 - 6.62)	0.010
NT-proBNP, pmol/L	13.47 (5.85 - 31.58)	15.97 (7.73 - 31.79)	-2.50 (-7.42 - 5.28)	0.457
CRP, mg/L	2.07 (0.88 - 4.80)	0.78 (0.38 - 1.68)	1.28 (0.69 - 2.12)	<0.001

CRP=C-reactive Protein; HDL=High-density Lipoprotein; LDL=Low-density Lipoprotein; NT-proBNP=N-terminal pro B-type Natriuretic Peptide. Data represent mean (95% confidence interval) biomarker values that were derived from nested linear mixed effects models, with adjustment for the use of cardiovascular medication, including aspirin, statins, beta-blockers, ACE-inhibitors or angiotensin-II receptor blockers, nitrates and anti-diabetics.

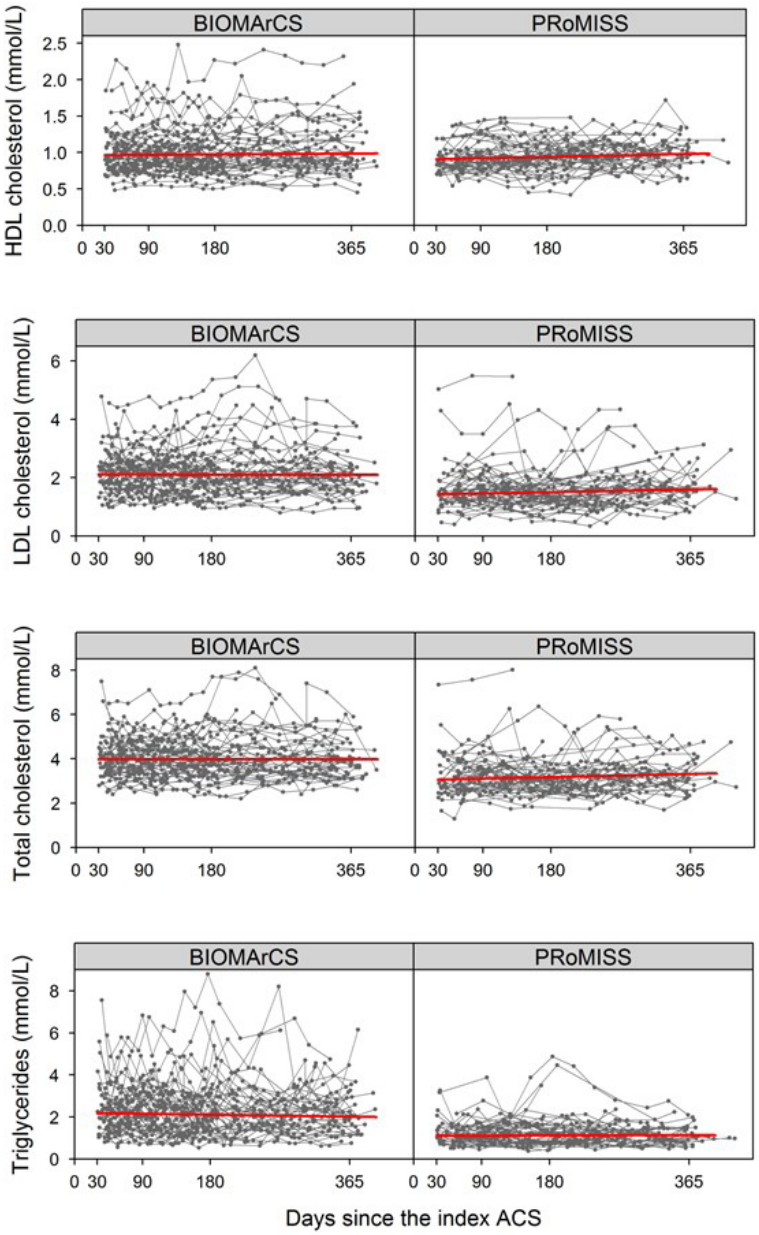


Figure 2. Serial measurements and temporal evolvement of lipid biomarkers in the BIOMArCS Caucasian and PRoMISS-Chinese patients.

HDL=high-density lipoprotein; LDL=low-density lipoprotein. The graphs show evolvement of HDL, LDL, total cholesterol and triglycerides >30 days since the index ACS event until 1-year in Caucasian (left) and Chinese (right) patients, who have not experienced a recurrent event during follow-up. The points represent measurements in individual patients. The bold (red) lines represent the average values, using linear mixed models with nested random effects.

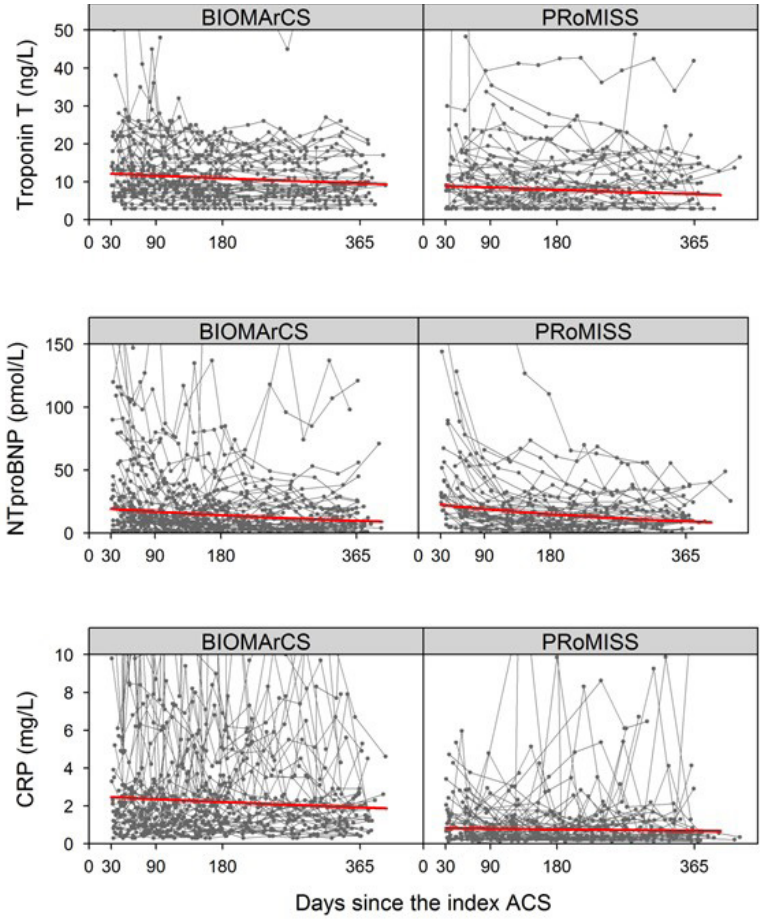


Figure 3. Serial measurements and temporal evolvement of acute-phase biomarkers in the BIOMArCS-Caucasian and PRoMISS-Chinese patients.

CRP=C-reactive Protein; NT-proBNP=N-Terminal fragment of Pro-Brain Natriuretic Peptide. The graphs show evolvement of Troponin T, NT-proBNP and CRP >30 days since the index ACS event until 1-year in Caucasian (left) and Chinese (right) patients, who have not experienced a recurrent event during follow-up. The points represent measurements in individual patients. The bold (red) lines represent the average values, using linear mixed models with nested random effects.

Discussion

This study investigated temporal cardiovascular biomarker profile differences between Caucasian and Chinese DM2 patients by high-frequency blood sampling during 1 year after their ACS index event. Overall, we found persistently higher levels of LDL, total cholesterol, triglycerides, TnT and CRP in Caucasian patients as compared to Chinese patients. We did not observe significant differences in HDL and NT-proBNP values between the two cohorts. In general, studies investigating inter-ethnic cardiovascular biomarker differences between Caucasian and Chinese patients in a CAD population have barely been performed. So far, only one systematic review has reported differences in ten conventional cardiovascular biomarkers between diverse ethnic Asian groups and Caucasians in the general population [9]. It is important to note that only 5 out of the 33 studied cohorts were from Chinese origin, of which only 1 resided in the country of origin. The vast majority of biomarker levels was described in South Asians, who are known to carry a more unfavourable cardiovascular risk and biomarker profile. Thus, sufficient evidence on inter-ethnic biomarker differences with data from Chinese CAD individuals is currently lacking. This underscores the need for ethnicity-driven biomarker research with a specific focus on Chinese individuals with CAD. Furthermore, the observed blood biomarker differences are based on only one blood sample, which reflects a snapshot and not a state during a longer period.

The importance of investigating this matter is endorsed by evidence that some biomarkers (e.g. CRP, IL-6, fibrinogen) were not able to predict incident CAD events risk among asymptomatic Chinese people in contrast to positive associations found in Caucasians [15]. Therefore, it seems inevitable to create ethnic-specific cut-off points in order to detect high-risk individuals for risk stratification. Although it has been demonstrated that these biomarkers retain their predictive value in Chinese cohorts despite their lower values, clear cut-off points are not known and should be further investigated. Also it is a matter of debate whether patients with lower values (e.g. LDL, CRP) would still benefit from treatment with anti-inflammatory agents or statins. For example, it seems that Asian patients are likely to benefit from lowering LDL by statins despite their lower values [16]. However, the threshold for treatment initiation and targets for treatment follow-up are possibly lower than for Caucasians. These thresholds and targets need to be determined in future studies as well.

With regard to our findings in the lipid profile, marked differences were found between Chinese and Caucasians during follow-up. Overall, except for HDL, average mean levels of

LDL, total cholesterol and triglycerides were significantly lower in Chinese than Caucasian patients. Differences in lipid profile among ethnic groups have been described by previous studies before and are in accordance with our results [17-19]. Especially Chinese have been pointed out to possess a favourable lipid profile [20]. An analysis from the INTERHEART study obtained one non-fasting blood sample from 5731 myocardial infarction patients and 6469 non-cardiac patients to investigate lipid abnormalities among Asian subgroups [18]. In particular, among the various Asian subgroups, Chinese patients tended to have the lowest LDL and triglycerides levels, but not HDL.

Further, our study showed that on the long run TnT and NT-proBNP levels varied in a similar range in the two cohorts and were not different from each other. This is in contrast to recent findings from the Multi-Ethnic Study of Atherosclerosis, where it has been demonstrated that Chinese individuals possessed the lowest NT-proBNP levels based upon genetics [21]. However, this study included asymptomatic individuals without prevalent cardiovascular disease and only a small proportion consisted of Chinese individuals (13%), in whom NT-proBNP levels were measured once at baseline. Further, ethnicity was self-reported, which may have resulted in misclassification. Altogether, the discrepancy with our findings could have been due to a different study population and design.

Lastly, remarkable differences regarding CRP in our study were present. The fact that Chinese patients had sustainably lower levels of CRP than Caucasians in our study is in accordance with existing evidence on CRP in various ethnic groups [22-26]. The underlying pathophysiological mechanism for lower CRP levels in Chinese individuals is unknown, but is speculated to be based upon differences in body mass index and genetics [22, 26]. Nevertheless, despite lower CRP levels, they still independently predict cardiovascular as well as all-cause mortality in Asian populations [24, 26].

Our study has several limitations. Firstly, due to border law regulations, blood samples could not be shipped from China and therefore needed to be analyzed in two separate laboratories. Thus, some amount of analytical variation differences was unavoidable and might have influenced our results. Secondly, with regard to our findings in the lipid profile, we accounted for differences in prevalence of statin use between BIOMArCS-Caucasians and PRoMISS-Chinese in the analysis, but we had no data on the (dynamic changes in) statin dosage. Nevertheless, from empiric data it is most likely that Caucasian patients were prescribed more often high-intensity statin therapy than Chinese patients, which emphasizes the importance of the observed differences in lipid values even more. Furthermore, we do

not have specific information on the 'clinical phenotype' of our studied patients, such as left ventricular ejection fraction, infarct size and severity of vessel disease, which might be confounders of the observed biomarker differences between the cohorts. However, by matching on clinical characteristics and admission diagnosis, we tried to limit this type of confounding. Another important limitation is the lack of information on genetic and environmental factors, since the observed differences could partly be due to divergent genetic makeup and different lifestyle (e.g. dietary factors, physical activity). Lastly, blood samples were not fasting samples. Nevertheless, HDL, LDL and total cholesterol are recognized as being relatively unaffected by the non-fasting state. Also, a non-fasting state reflects a state in which patients often present in the hospital and thus mimics clinical practice.

Conclusion

Frequent blood sampling during 1 year post ACS enabled us to reveal that most conventional biomarkers were remarkably lower in diabetic CAD participants from Chinese than Caucasian origin. This could give more insight into blood biomarker related differences among ethnic groups and might serve as a reference pilot study for larger future CAD studies. Our findings underscore the fact that it may not be convenient to apply findings from most Western cohorts to Chinese individuals. In order to provide accurate risk stratification for prediction and treatment benefit, further research should focus on defining clear cut-off values in primary and secondary prevention for each specific ethnic group.

References

1. Roth G.A. et al. Global, Regional, and National Burden of Cardiovascular Diseases for 10 Causes, 1990 to 2015. J Am Coll Cardiol 2017; 70: 1-25.
2. Shepard D. et al. Ischemic Heart Disease Worldwide, 1990 to 2013: Estimates From the Global Burden of Disease Study 2013. Circ Cardiovasc Qual Outcomes 2015; 8: 455-456.
3. Joseph P. et al. Reducing the Global Burden of Cardiovascular Disease, Part 1: The Epidemiology and Risk Factors. Circ Res 2017; 121: 677-694.
4. Roth G.A. et al. Demographic and epidemiologic drivers of global cardiovascular mortality. N Engl J Med 2015; 372: 1333-1341.
5. Reddy K.S. Cardiovascular disease in non-Western countries. N Engl J Med 2004; 350: 2438-2440.
6. Wang J. et al. Novel biomarkers for cardiovascular risk prediction. J Geriatr Cardiol 2017; 14: 135-150.
7. He L.P. et al. Early C-reactive protein in the prediction of long-term outcomes after acute coronary syndromes: a meta-analysis of longitudinal studies. Heart 2010; 96: 339-346.
8. Cheng J.M. et al. Evaluation of 42 cytokines, chemokines and growth factors for prediction of cardiovascular outcome in patients with coronary artery disease. Int J Cardiol 2015; 184: 724-727.
9. Gijsberts C.M. et al. Biomarkers of Coronary Artery Disease Differ Between Asians and Caucasians in the General Population. Glob Heart 2015; 10: 301-311 e11.
10. Li H. and Ge J. Cardiovascular diseases in China: Current status and future perspectives. Int J Cardiol Heart Vasc 2015; 6: 25-31.
11. Wang L. et al. Prevalence and Ethnic Pattern of Diabetes and Prediabetes in China in 2013. Jama, 2017; 317: 2515-2523.
12. Chan J.C. et al. Diabetes in Asia: epidemiology, risk factors, and pathophysiology. Jama 2009; 301: 2129-2140.
13. Hu G. et al. Sex differences in cardiovascular and total mortality among diabetic and non-diabetic individuals with or without history of myocardial infarction. Diabetologia 2005; 48: 856-861.
14. Oemrawsingh R.M. et al. Cohort profile of BIOMArCS: the BIOMarker study to identify the Acute risk of a Coronary Syndrome-a prospective multicentre biomarker study conducted in the Netherlands. BMJ Open 2016; 6: e012929.
15. Veeranna V. et al. Association of novel biomarkers with future cardiovascular events is influenced by ethnicity: results from a multi-ethnic cohort. Int J Cardiol 2013; 166: 487-493.
16. Sakamoto T. and Ogawa H. “Just make it lower” is an alternative strategy of lipid-lowering therapy with statins in Japanese patients: LDL-cholesterol: the lower, the better; is it true for Asians? (Con). Circ J 2010; 74: 1731-1741.
17. Frank A.T. et al. Racial/ethnic differences in dyslipidemia patterns. Circulation 2014; 129: 570-579.
18. Karthikeyan G. et al. Lipid profile, plasma apolipoproteins, and risk of a first myocardial infarction among Asians: an analysis from the INTERHEART Study. J Am Coll Cardiol 2009; 53: 244-253.
19. Goff D.C. Jr. et al. Dyslipidemia prevalence, treatment, and control in the Multi-Ethnic Study of Atherosclerosis (MESA): gender, ethnicity, and coronary artery calcium. Circulation 2006; 113: 647-656.
20. Anand S.S. et al. Differences in risk factors, atherosclerosis, and cardiovascular disease between ethnic groups in Canada: the Study of Health Assessment and Risk in Ethnic groups (SHARE). Lancet 2000; 356: 279-284.
21. Gupta D.K. et al. Differences in Natriuretic Peptide Levels by Race/Ethnicity (From the Multi-Ethnic Study of Atherosclerosis). Am J Cardiol 2017; 120: 1008-1015.
22. Kelley-Hedgepeth A. et al. Ethnic differences in C-reactive protein concentrations. Clin Chem 2008; 54: 1027-

1037.

23. Lakoski S.G. et al. Gender and C-reactive protein: data from the Multiethnic Study of Atherosclerosis (MESA) cohort. Am Heart J 2006; 152: 593-598.
24. Sung K.C. et al. C-reactive protein and risk of cardiovascular and all-cause mortality in 268 803 East Asians. Eur Heart J 2014; 35: 1809-1816.
25. Saito I. et al. A low level of C-reactive protein in Japanese adults and its association with cardiovascular risk factors: the Japan NCVC-Collaborative Inflammation Cohort (JNIC) study. Atherosclerosis 2007; 194: 238-244.
26. Saito I. et al. C-reactive protein and cardiovascular disease in East asians: a systematic review. Clin Med Insights Cardiol 2014; 8: 35-42.

Supplementary Table 1. Estimated coefficients describing the temporal evolution of selected biomarkers in the BIOMArCS and PRoMISS cohorts 30 days to 1 year after the index ACS (models with Cohort*Time interaction).

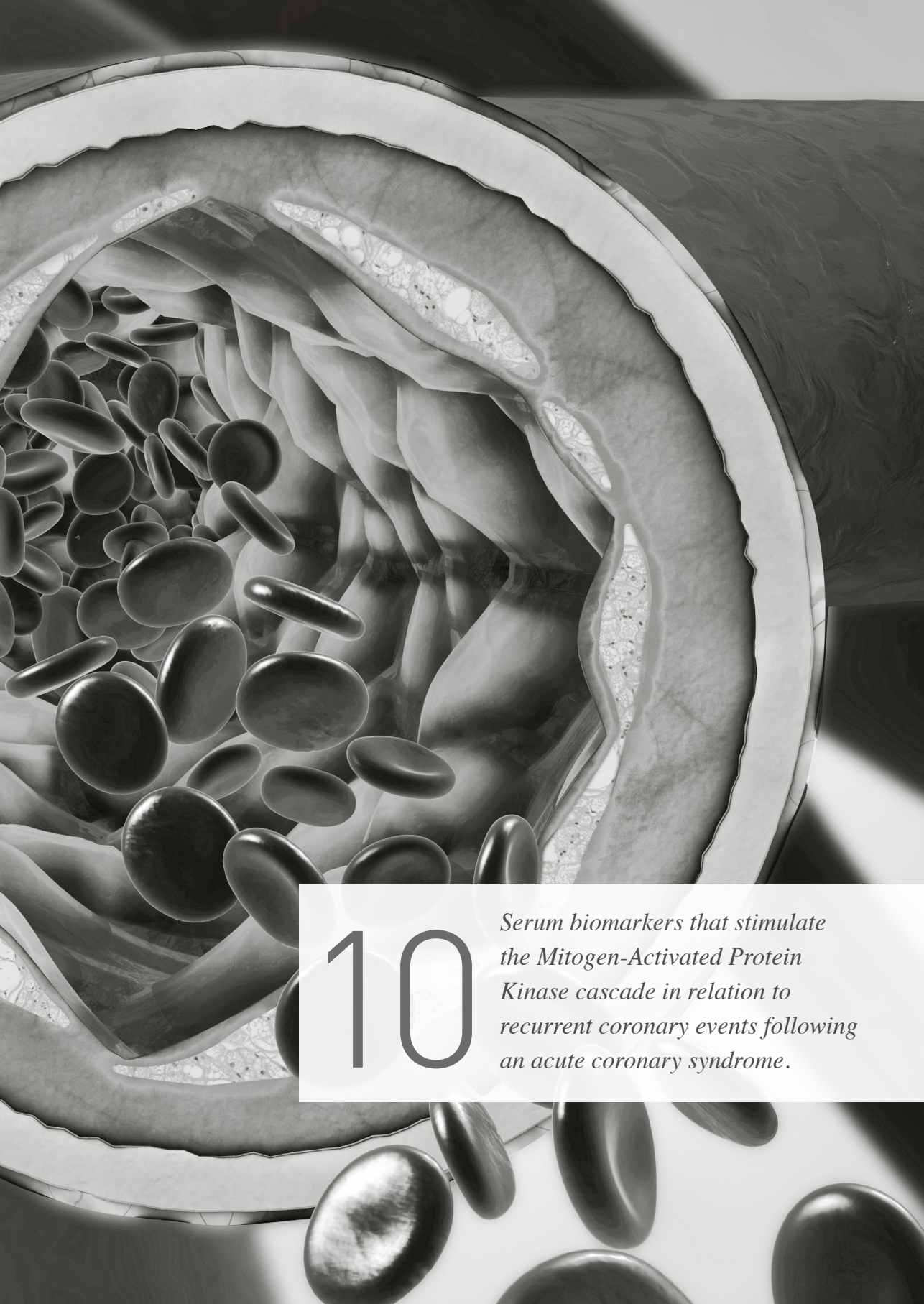
	PRoMISS					BIOMArCS					Cohort*Time interaction
	Mean at Time = 0		Time			Mean at Time = 0		Time			
	β_0	SE	β_2	SE	P-value	$\beta_0 + \beta_1$	SE	$\beta_2 + \beta_3$	SE	P-value	P-value of β_3
HDL, log2 (mmol/L)	-0.233	0.077	0.010	0.003	<0.001	-0.147	0.086	0.003	0.002	0.183	0.035
LDL, log2 (mmol/L)	0.597	0.124	0.013	0.005	0.006	1.221	0.139	0.001	0.004	0.816	0.043
Cholesterol, log2 (mmol/L)	1.694	0.078	0.009	0.003	0.001	2.131	0.088	0.001	0.002	0.536	0.032
Triglycerides, log2 (mmol/L)	0.336	0.154	0.004	0.006	0.576	1.422	0.174	-0.007	0.005	0.139	0.177
Troponin T, log2 (ng/L)	3.339	0.220	-0.036	0.008	< 0.001	0.381	0.246	-0.028	0.006	0.022	0.459
NT-proBNP, log2 (pmol/L)	4.662	0.362	-0.120	0.012	< 0.001	4.184	0.400	-0.077	0.009	<0.001	0.004
CRP, log2 (mg/L)	-0.768	0.348	-0.029	0.015	0.054	0.691	0.393	-0.040	0.012	<0.001	0.561

ACS= acute coronary syndrome; CRP = C-reactive Protein; HDL = High-density Lipoprotein; LDL = Low-density Lipoprotein; NT-proBNP = N-terminal pro B-type Natriuretic Peptide; SE = standard error. Data represent regression coefficients of nested linear mixed effects models. The basic structure of the models was: log2(biomarker) = $\beta_0 + \beta_1$*Cohort + β_2*Time + β_3*Cohort*Time, with Cohort (BIOMArCS=1 versus PRoMISS=0), Time (months since index ACS) and Cohort*Time modelled as fixed effects, and paired patients (BIOMArCS-PRoMISS) as random effects. Regression coefficients were adjusted for the use of cardiovascular medication, including aspirin, statins, beta-blockers, ACE-inhibitors or angiotensin-II receptor blockers, nitrates and anti-diabetics.

Supplementary Table 2. Estimated coefficients describing the temporal evolution of selected biomarkers in the BIOMArCS and PRoMISS cohorts 30 days to 1 year after the index ACS (models without Cohort*Time interaction).

	Mean in PRoMISS at Time = 0		Mean in BIOMArCS at Time = 0		Mean difference between BIOMArCS and PRoMISS, irrespective of Time			Time		
	β_0	SE	$\beta_0 + \beta_1$	SE	β_1	SE	P-value	β_2	SE	P-value
HDL, log2(mmol/L)	-0.208	0.076	-0.161	0.086	0.047	0.068	0.491	0.006	0.002	<0.001
LDL, log2(mmol/L)	0.638	0.122	1.198	0.138	0.560	0.106	<0.001	0.005	0.003	0.055
Cholesterol, log2(mmol/L)	1.721	0.077	2.115	0.087	0.393	0.066	<0.001	0.005	0.002	0.012
Triglycerides, log2(mmol/L)	0.374	0.152	1.401	0.174	1.027	0.121	<0.001	-0.003	0.004	0.415
Troponin T, log2(ng/L)	3.313	0.217	3.822	0.245	0.509	0.189	0.010	-0.031	0.005	<0.001
NT-proBNP, log2(pmol/L)	4.514	0.360	4.267	0.400	-0.245	0.327	0.457	-0.094	0.007	<0.001
CRP, log2(mg/L)	-0.731	0.342	0.667	0.390	1.399	0.243	<0.001	-0.035	0.009	<0.001

ACS = acute coronary syndrome; CRP = C-reactive Protein; HDL = High-density Lipoprotein; LDL = Low-density Lipoprotein; NT-proBNP = N-terminal pro B-type Natriuretic Peptide; SE = standard error. Data represent regression coefficients of nested linear mixed effects models. The basic structure of the models was: log2(biomarker) = $\beta_0 + \beta_1$*Cohort + β_2*Time, with Cohort (BIOMArCS=1 versus PRoMISS=0) and Time (months since index ACS) modelled as fixed effects, and paired patients (BIOMArCS-PRoMISS) as random effects. Regression coefficients were adjusted for the use of cardiovascular medication, including aspirin, statins, beta-blockers, ACE-inhibitors or angiotensin-II receptor blockers, nitrates and anti-diabetics.



10

Serum biomarkers that stimulate the Mitogen-Activated Protein Kinase cascade in relation to recurrent coronary events following an acute coronary syndrome.

10

Serum biomarkers that stimulate the Mitogen-Activated Protein Kinase cascade in relation to recurrent coronary events following an acute coronary syndrome.

Vroegindewey MM, Buljubasic N, Oemrawsingh RM, Kardys I, Asselbergs FW, van der Harst P, Umans VA, Kietselaer B, Lenderink T, Liem A, Mouthaan H, Boersma E, Akkerhuis KM.

J Mol Biomark Diagn 2019; 10(2): 414. doi: 10.4172/2155-9929.1000414.

Abstract

Rationale: The intracellular mitogen-activated protein kinase (MAPK) cascade regulates intracellular processes that modulate cardiovascular disease progression. We explored the time-course of serum biomarkers that stimulate the MAPK-cascade in post-acute coronary syndrome (ACS) patients, prior to a recurrent coronary event.

Methods & Results: BIOMArCS is a high-frequent repeated blood sampling study in post-ACS patients. We performed a nested case-control study selecting the 45 patients who experienced a recurrent event (cases) and 2 matched event-free controls per case during 1-year follow-up. Olink Proteomics 'immunoassay was used to measure 25 serum biomarkers. Results are expressed in the arbitrary Normalized Protein eXpression (NPX) unit on the 2logscale. Linear mixed-effects models were applied to examine time-courses and differences between cases and controls. Mean age was 66 ± 12 years and 80% were men, with no differences between cases and controls. Early cases had significantly higher levels of ANG-1 (difference 0.95 NPX (95% CI 0.36-1.54), PAR-1 (difference 0.50 NPX

(95% CI 0.22-0.77) and BMP-6 (difference 0.55 NPX (95% CI 0.21-0.90) than controls. No differences in biomarker levels were observed between late cases and matching controls. In particular, in cases, no increase was observed prior to the moment of the recurrent event.

Conclusions: Patients with an early recurrent coronary event after an index-ACS had higher levels of ANG-1, PAR-1 and BMP-6 than patients who remained event-free.

Introduction

Although outcome for patients with cardiovascular disease (CVD) has improved over the last decades, hospitalization rates are still increasing [1-3]. This increase can partly be explained by the increasing population of patients who survived an acute coronary syndrome (ACS) and who are at risk of experiencing a recurrent coronary event [2]. As CVD is dynamic and shows considerable interpatient variation, to improve secondary prevention, insight in the course of CVD in individual subjects is required. Since biomarker profiles may serve as a proxy for CVD status and development, the exploration of (established and) evolving markers covering relevant pathophysiological processes is warranted.

The mitogen-activated protein kinase (MAPK) cascade, is an intracellular cascade of proteins that enables extracellular stimuli - blood biomarkers - to modulate several intracellular processes i.e., cell growth, differentiation, proliferation, and apoptosis [4]. Basic research has shown that the MAPK-cascade plays a pivotal role in cellular processes that advance CVD progression [5]. First, the MAPK-cascade promotes atherosclerotic lesion formation, i.e., by inducing inflammation and cell apoptosis. Secondly, it may activate pathological cardiac remodeling after myocardial infarction by constraining myocyte mitosis and promoting fibrosis. Thirdly, the cascade might be directly involved in the development of in-stent restenosis [5].

In view of the pivotal role of the MAPK-cascade in the development and progression of CVD, blood biomarkers that regulate the cascade may be useful for the identification of patients with CVD who are at higher risk of developing a (recurrent) coronary event. However, translational research relating the MAPK-cascade to clinical CVD progression is scarce [5].

We aimed to explore the course of protein blood biomarkers that stimulate the intracellular MAPK-cascade in post-ACS patients prior to the development of a recurrent coronary event during one year of follow-up.

Methods

Study population

We performed a case-control study that is embedded in The 'BIOMarker study to identify the Acute risk of a Coronary Syndrome' (BIOMArCS) [6]. BIOMArCS is a multicenter observational study with a unique high-frequency sampling design, to study the course of blood biomarkers in patients following an ACS in anticipation of a recurrent event. The design of BIOMArCS has been described in detail elsewhere [6]. In brief, BIOMArCS enrolled 844 patients with ACS, aged ≥ 40 years and who had at least 1 pre-specified cardiovascular risk factor. After enrolment, venipuncture was performed at admission, discharge, and subsequently every two weeks during the first half-year and every month during the second half-year. A median number of 17 repeated blood samples per patient were obtained.

BIOMArCS was approved by the Institutional Review Boards of all enrolling hospitals, and all participating patients provided written informed consent. BIOMArCS is registered in The Netherlands Trial Register NTR1698 and NTR1106.

Case-control design

The current analysis is based on a case-control approach. A total of 45 patients (cases) in BIOMArCS reached the composite study endpoint of cardiac death, non-fatal myocardial infarction (MI), or unstable angina (UA) requiring urgent coronary revascularization during one year of follow-up after the index-ACS. These cases were with two controls that are selected from BIOMArCS event-free patients. Cases and controls were matched on age, sex and admitted hospital. For reasons of efficiency, for each case, the blood sample at hospital admission and the last and second last samples prior to the recurrent coronary event have been analyzed. In controls, we selected the blood sample at hospital admission and the blood sample that corresponds in time with the recurrent event of the matched case.

As a pragmatic choice, separate analyses were performed for cases (and their matching controls) that experienced their event in the first 30 days after the index-ACS, and for cases (and their matching controls) that experienced their event thereafter. Hence, we were able to differentiate between the behavior of biomarkers during the acute and post-stabilization phase after the index-ACS.

Biomarker measurements

Targeted protein biomarker measurements were performed by the Proximity Extension Assay (PEA) Technique using Olink Proteomics' CVD II panel (Olink Proteomics AB, Uppsala, Sweden). Details concerning PEA and the CVD II panel are described on the website of Olink Proteomics (www.olink.com). In brief, the PEA technique consists of a pair of oligonucleotide-labelled antibody probes that pairwise bind to a targeted protein biomarker in a blood sample. This binding induces amplification of the protein biomarker by real-time PCR (Fluidigm® BioMark™ HD System).

The PEA technique enables simultaneous analysis of all protein biomarkers of the CVD II panel in one blood sample. Olink proteomics' CVD II panel provides measurements of 25 protein blood biomarkers that are related to the intracellular MAPK-cascade (overview of proteins in Table 1). Every measured protein blood biomarker is expressed in an arbitrary unit on the log2-scale called Normalized Protein eXpression (NPX). Accordingly, an increase or decrease of one NPX corresponds with a doubling or halving of a biomarker serum level. NPX values cannot be compared across different proteins. For each protein biomarker, general calibrator curves to calculate approximate concentrations are available on the website of Olink Proteomics.

Table 1. Overview of the assessed protein biomarker.

Abbreviation	Full name	Synonyms	Molecular function
NEMO	NF-kappa-B essential modulator	IKBKG, FIP3	Binding protein
HB-EGF	Proheparin-binding EGF-like growth factor	DTR, DTS, HEGFL	Growth factor/receptor
SCF	Stem cell factor	KITLG,MGF	Cytokine/growth factor
PDGF subunit B	Platelet-derived growth factor subunit B	PDGFB,PDGF2, SIS	Developmental protein/ growth factor
GDF-2	Growth/differentiation factor 2	BMP9	Cytokine/growth factor
ANG-1	Angiopoietin-1	ANGPT1	Developmental protein
CCL3	C-C motif chemokine 3	MIP1A, SCYA3, G0S19-1	Chemokine
TIE2	Angiopoietin-1 receptor	TEK, VMCM, VMCM1	Receptor
PAR-1	Proteinase-activated receptor 1	F2R, CF2R, TR	Receptor
LEP	Leptin	OB, OBS	Hormone/growth factor
REN	Renin		Hydrolase
TNFRSF11A	Tumor necrosis factor receptor superfamily member 11A	RANK, ODFR, CD265, NFKB activator	Receptor
THPO	Thrombopoietin / megakaryocyte colony-stimulating factor	MGDF	Cytokine/hormone
FGF-21	Fibroblast growth factor 21		Growth factor
GAL-9	Galectin-9	LGALS9	Binding protein

Table 1 continued.

Abbreviation	Full name	Synonyms	Molecular function
SRC	Proto-oncogene tyrosine-protein kinase SRC	SRC1, C-SRC	Kinase
GH	Growth hormone	GH1, somatotropin	Hormone
XCL1	X-C motif chemokine ligand 1	Lymphotactin, LTN, SCYC1, ATAC, SCM-1	Chemokine
FGF-23	Fibroblast growth factor 23	HYPF	Growth factor
CCL17	C-C motif chemokine 17	SCYA17, TARC	Chemokine
IL-18	Interleukin-18	IGIF, IL1F4	Cytokine
BMP-6	Bone morphogenetic protein 6	VGR1, VGR	Cytokine/developmental protein/growth factor
IL-6	Interleukin-6	IFNB2, BSF2, CDF, HGF	Cytokine/growth factor
AMBP	Alpha-1-microglobulin/bikunin precursor	HCP, ITIL, ITI, Bikunin, EDC1, Trypstatin	Protease inhibitor
CD40-L	CD40 ligand	CD40LG, TNFSF5, TRAP, HIGM1, CD154	Cytokine

Statistical analysis

Continuous variables are presented as medians with interquartile range (IQR) and categorical variables as numbers with percentages. Differences between cases and controls were compared with Mann-Whitney U and Pearson Chi-square tests, respectively.

As indicated, all biomarkers were analyzed on a log2-transformed scale. We fitted a linear mixed-effects model for every biomarker to describe patient-specific longitudinal biomarker trajectories as a function of time. Likelihood ratio tests and F-tests were used for hypothesis testing, whereas residuals were used to examine the model assumptions.

We considered 25 biomarkers. To adjust for inflation of the type I error with multiple testing, statistical significance was stated at p=0.003 (two-tailed test), based on the matrix spectral decomposition method [7]. R statistical software (version 3.4.0) was used for analyses, in particular the package nlme (https://cran.r-project.org/web/packages/nlme/index.html).

Results

Baseline characteristics

Mean age of all patients was 66.0 ± 11.9 years and 80.2% were men. Cases and controls did not show any significant differences in presentation, initial treatment, cardiovascular risk factors and medication at first blood sample, indicating successful matching (Table 2).

Table 2. Baseline clinical characteristics.

	Cases (n = 44)	Controls (n = 87)	P-value
Presentation and initial treatment			
Age, years	67.5 (57.3-77.5)	66.7 (57.4-75.5)	0.83
Male gender, n (%)	35 (79.5)	70 (80.5)	0.90
Admission diagnosis, n (%)			0.38
STEMI	16 (36.4)	42 (48.3)	
NSTEMI	22 (50.0)	33 (37.9)	
UAP	6 (13.6)	12 (13.8)	
CAG performed, n (%)	39 (88.6)	82 (94.3)	0.25
PCI performed, n (%)	33 (86.8)	67 (81.7)	0.48
CKmax, U/L	418 [195-1142]	513 [169-1332]	0.94
Cardiovascular risk factors			
Smoking, n (%)			0.81
Current	17 (38.6)	35 (40.2)	
Former	12 (27.3)	27 (31.0)	
Never	15 (34.1)	25 (28.7)	
Diabetes mellitus	16 (36.4)	32 (36.8)	0.96
Hypertension, n (%)	21 (47.7)	44 (50.6)	0.76
Hypercholesterolemia, n (%)	19 (43.2)	46 (52.9)	0.30
Creatinine, μmol/L	88 (73-93)	81 (67-97)	0.15
Cardiovascular history			
Peripheral arterial disease, n (%)	10 (22.7)	7 (8.0)	0.018
Myocardial infarction, n (%)	14 (31.8)	33 (37.9)	0.49
Percutaneous coronary intervention, n (%)	14 (31.8)	29 (33.3)	0.86
Coronary artery bypass grafting, n (%)	10 (22.7)	17 (19.5)	0.67
Stroke, n (%)	9 (20.5)	5 (5.7)	0.010
Valvular heart disease, n (%)	4 (9.1)	3 (3.4)	0.18
Heart failure, n (%)	4(9.1)	1 (1.1)	0.025
Medication at first blood sample moment >7 days after the index ACS[a]			
Aspirin, n (%)	35 (92.1)	76 (92.7)	0.91
P2Y12 inhibitor, n (%)	36 (94.7)	74 (90.2)	0.41
Vitamin K antagonist, n (%)	7 (18.4)	8 (9.8)	0.18
Statin, n (%)	35 (92.1)	79 (96.3)	0.32
Beta-blocker, n (%)	36 (94.7)	69 (84.1)	0.10
ACE-inhibitor or ARB, n (%)	34 (89.5)	65 (79.3)	0.17

ACE=angiotensin converting enzyme; ARB=angiotensin II receptor blocker; CKmax=maximum creatine kinase during the index admission; NSTEMI=non-ST-elevation myocardial infarction; STEMI: ST-elevation myocardial infarction; UAP: unstable angina pectoris.[a]The first blood sample >7 days was taken at a median (25th-75th percentile) of 24 (16-34) days after the index ACS. Continuous variables are presented as median [25th-75th percentile]. Categorical variables are presented as number (percentage).

Biomarker trajectories in the first 30 days after the index ACS

Fifteen cases reached the study endpoint within 30 days after the index ACS. They had higher serum levels of ANG-1 (difference of 0.95 NPX, 95% confidence interval (CI) 0.36-1.54), PAR-1 (difference of 0.50 NPX, 95% CI 0.22-0.77) and BMP-6 (difference of 0.55 NPX, 95% CI 0.21-0.90) than the matched controls (Table 3, Figure 1 left hand panel).

Table 3. Difference in biomarker serum level between cases and controls ≤ 30 days.

Biomarker (NPX)	Coefficient	95% CI	P-value
NEMO	1.16	(0.36 - 1.95)	0.005
HB-EGF	0.61	(0.11 - 1.12)	0.018
SCF	-0.13	(-0.59 - 0.32)	0.55
PDGF subunit B	0.92	(0.31 - 1.54)	0.004
GDF-2	-0.063	(-0.35 - 0.23)	0.66
ANG-1	0.95	(0.36 - 1.54)	0.002
CCL3	0.29	(-0.12 - 0.71)	0.16
TIE2	0.12	(-0.048 - 0.29)	0.16
PAR-1	0.50	(0.22 - 0.77)	<0.001
LEP	0.35	(-0.31 - 1.00)	0.29
REN	0.45	(-0.18 - 1.08)	0.16
TNFRSF11A	0.41	(0.003 - 0.82)	0.048
THPO	0.22	(-0.018 - 0.45)	0.069
FGF-21	0.55	(-0.39 - 1.49)	0.24
GAL-9	0.16	(-0.083 - 0.41)	0.19
SRC	0.29	(-0.18 - 0.76)	0.22
GH	0.71	(-0.61 - 2.02)	0.29
XCL1	0.34	(0.018 - 0.66)	0.039
FGF-23	0.90	(0.16 - 1.63)	0.018
CCL17	0.77	(0.089 - 1.46)	0.028
IL-18	0.19	(-0.15 - 0.52)	0.27
BMP-6	0.55	(0.21 - 0.90)	0.002
IL-6	0.95	(0.074 - 1.82)	0.034
AMBP	0.11	(-0.033 - 0.25)	0.13
CD40-L	1.32	(0.41 - 2.22)	0.006

ACS=acute coronary syndrome, CI=confidence interval, NPX=Normalized Protein eXpression. For every biomarker, the difference in biomarker serum level between cases and controls is expressed in a relative arbitrary unit on the log 2 scale. Thus, an increase or decrease of one NPX corresponds with a doubling or a halving of the protein biomarker serum level.

Biomarker trajectories after 30 days

Thirty cases had the study endpoint in >30 days after the index ACS. Interestingly, in these late cases, the biomarker levels during the first 30 days after the index ACS tended to be lower than in the early cases (Table 4). In the post-30 day time window, cases and matched controls appeared to have similar levels of biomarkers (Table 5). Importantly, we found no steady or sudden increase in biomarkers in the days or weeks prior to the recurrent event.

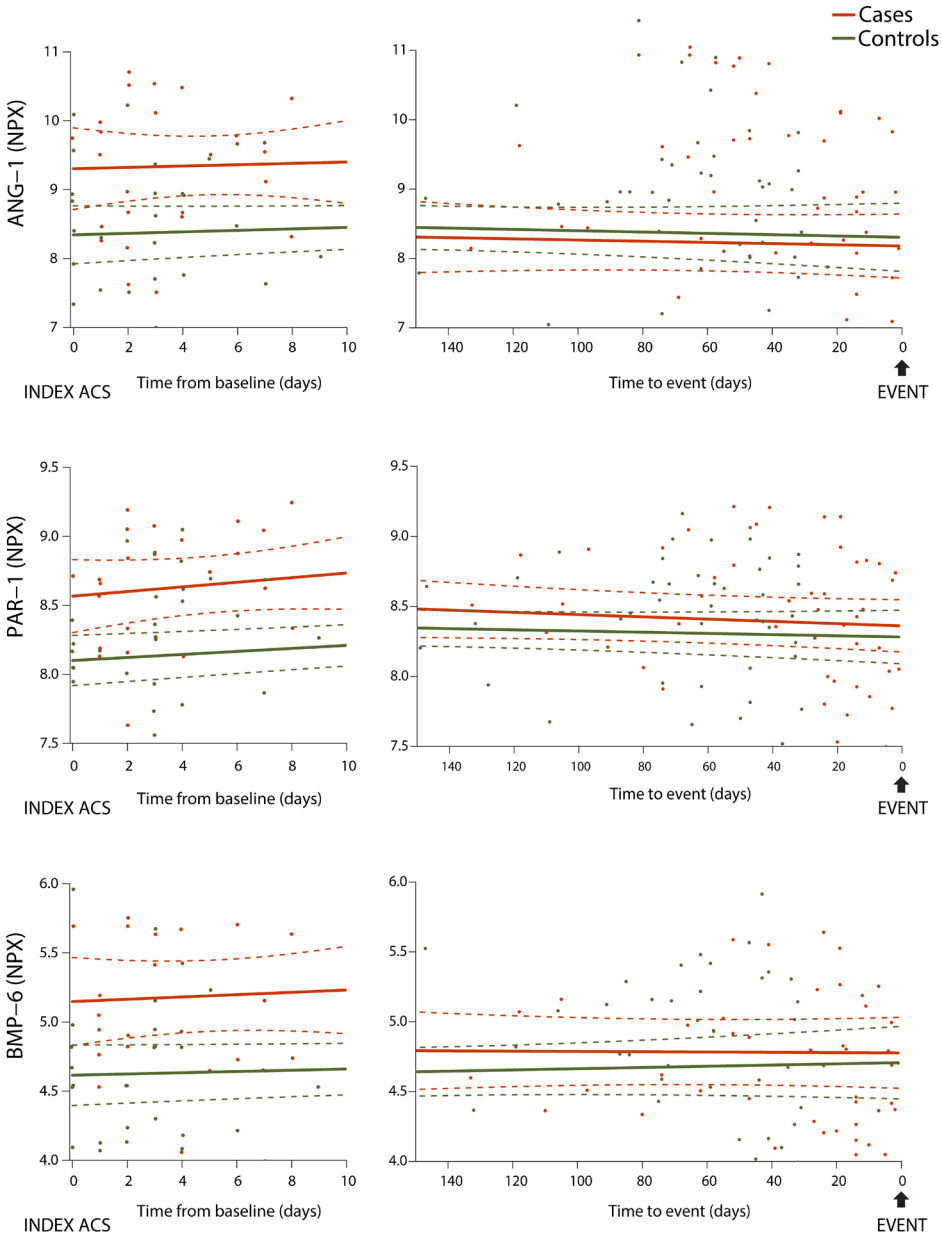


Figure 1. Timecourse of PAR-1, BMP-6 and ANG-1.

NPX=Normalized Protein eXpression.

Table 4. Biomarker serum levels in the first 30 days for cases only.

Biomarker (NPX)	Early cases[a]	Late cases[a]	P-value
NEMO	6.61±1.08	5.80±1.25	0.062
HB-EGF	5.62±0.89	5.03±0.73	0.049
SCF	8.31±0.87	8.08±0.60	0.40
PDGF subunit B	10.47±0.84	9.49±1.16	0.012
GDF-2	3.16±0.55	2.94±0.37	0.19
ANG-1	9.31±0.91	8.38±1.21	0.022
CCL3	4.04±0.59	3.59±0.36	0.013
TIE2	7.02±0.19	6.86±0.39	0.16
PAR-1	8.66±0.35	8.26±0.48	0.012
LEP	5.26±0.93	4.94±0.51	0.23
REN	8.36±1.34	8.31±1.06	0.91
TNFRSF11A	5.55±0.68	5.15±0.61	0.084
THPO	2.99±0.33	2.87±0.80	0.60
FGF-21	7.01±1.51	6.72±1.12	0.54
Gal-9	7.93±0.39	7.79±0.39	0.34
SRC	7.20±0.37	7.06±0.55	0.40
GH	8.02±2.14	7.27±1.96	0.31
XCL1	4.77±0.52	4.79±0.49	0.92
FGF-23	4.02±1.77	2.84±0.63	0.025
CCL17	8.74±1.14	8.57±1.26	0.70
IL-18	8.59±0.58	8.36±0.46	0.21
BMP-6	5.23±0.60	4.80±0.77	0.091
IL-6	6.00±1.86	4.89±1.37	0.062
AMBP	6.00±0.22	5.90±0.23	0.26
CD40-L	7.28±1.41	6.28±1.44	0.058

NPX: Normalized Protein eXpression**.** [a]Patient-level mean value±standard deviation. Blood samples ≤30 days after the index ACS were available for 15 ≤30 days cases and 17 >30 days cases.

Table 5. Biomarker serum levels in relation to time post index-ACS.

	Median maximum value ≤7 days[a]		Patient-level mean value ≤30 days[b]		Patient-level mean value >30 days[b]	
Biomarker (NPX)	**Cases**	**Controls**	**Cases**	**Controls**	**Cases**	**Controls**
NEMO	6.50 (5.51-7.29)	5.75 (4.70-6.63)	6.18 ±1.22	5.67±1.33	5.44±1.58	5.66±1.50
HB-EGF	5.43 (4.73-5.82)	5.06 (4.48-5.60)	5.31±0.85	5.15±0.88	5.19±0.89	5.32±0.95
SCF	8.18 (7.45-8.88)	8.35 (7.99-8.77)	8.19±0.74	8.40±0.54	8.45±0.55	8.53±0.45
PDGF subunit B	10.66 (9.37-10.87)	10.39 (8.95-10.69)	9.95±1.12	9.72±1.20	9.37±1.45	9.72±1.34
GDF-2	3.33 (3.74-3.59)	3.26 (2.97-3.60)	3.04±0.47	3.28±0.41	3.43±0.48	3.49±0.42
ANG-1	9.51 (7.86-9.78)	8.75 (7.72-9.54)	8.81±1.17	8.52±1.16	8.20±1.25	8.51±1.19
CCL3	3.97 (3.48-4.22)	3.55 (3.21-4.12)	3.80±0.52	3.70±0.68	3.59±0.55	3.62±0.52
TIE2	7.01 (6.89-7.19)	6.88 (6.71-7.17)	6.93±0.32	6.93±0.31	7.01±0.27	6.96±0.32
PAR-1	8.69 (8.27-8.88)	8.36 (7.88-8.59)	8.45±0.47	8.25±0.51	8.40±0.48	8.37±0.51
LEP	5.09 (4.58-5.73)	5.00 (3.96-5.79)	5.09±0.74	4.90±1.22	5.06±0.86	4.96±1.09
REN	8.74 (7.21-9.88)	7.88 (7.05-8.75)	8.34±1.18	8.08±1.06	8.47±1.01	8.24±1.00
TNFRSF11A	5.44 (5.04-6.01)	5.10 (4.60-5.51)	5.34±0.67	5.10±0.69	5.22±0.66	5.09±0.59
THPO	3.01 (2.75-3.42)	2.66 (2.35-2.97)	2.93±0.62	2.72±0.48	2.70±0.65	2.67±0.44
FGF-21	7.33 (6.71-8.36)	6.36 (5.26-7.59)	6.86±1.31	6.60±1.51	7.14±1.21	6.48±1.70
GAL-9	7.94 (7.64-8.10)	7.78 (7.42-8.05)	7.86±0.39	7.79±0.45	7.86±0.37	7.84±0.40
SRC	7.31 (7.21-7.44)	7.34 (7.00-7.61)	7.13±0.47	7.02±0.83	6.77±0.99	6.92±0.93
GH	9.07 (6.46-9.76)	7.15 (5.93-8.92)	7.62±2.05	7.52±2.01	7.32±1.92	7.72±2.05
XCL1	4.72 (4.50-5.21)	4.49 (4.19-4.87)	4.78±0.50	4.53±0.55	4.73±0.57	4.60±0.55
FGF-23	3.32 (2.78-3.89)	2.80 (2.55-3.24)	4.02±1.77	2.93±0.71	3.18±0.81	3.03±0.70
CCL17	8.53 (8.21-9.64)	8.12 (7.57-8.97)	8.74±1.14	8.32±1.39	8.47±1.30	8.46±1.29
IL-18	8.67 (8.07-9.00)	8.50 (7.97-8.76)	8.59±0.58	8.44±0.59	8.37±0.41	8.50±0.56
BMP-6	4.96 (4.65-5.69)	4.61 (4.14-5.11)	5.23±0.60	4.68±0.59	4.77±0.69	4.67±0.62
IL-6	6.56 (4.42-7.18)	5.08 (4.24-5.75)	6.00±1.86	4.63±1.42	3.99±1.04	3.69±0.91
AMBP	5.99 (5.82-6.18)	5.94 (5.68-6.07)	5.99±0.22	5.87±0.24	5.97±0.22	5.89±0.22
CD40-L	7.01 (6.17-8.31)	6.31 (5.40-7.29)	7.28±1.41	6.75±1.49	6.11±1.62	6.37±1.66

NPX=Normalized Protein eXpression. [a]Median (25th-75th percentile) value of the patient-level maximum. [b]Mean ± standard deviation value of the patient-level mean. Blood samples ≤7, ≤30 and >30 days after the index ACS were available for 23, 32, 28 cases and for 44, 67, 70 controls, respectively.

Discussion

Serum levels of ANG-1, PAR-1 and BMP-6 were significantly higher in patients who developed a recurrent coronary event within the first 30 days following an ACS than in their matching controls. In the time period >30 days after the index-ACS until 1 year follow-up, patients with and without a recurrent coronary event had similar patterns of MAPK stimulating biomarkers.

Our study results suggest that during the first 30 days post-ACS, the initial ACS induces numerous stimuli that activate the intracellular MAPK-cascade, which, in turn, may induce a pro-inflammatory and thrombogenic state, leading to a recurrent event. Potentially, other processes play a more important role in the initiation of a new coronary event following stabilization after the first 30 days post-ACS.

PAR-1 is a receptor expressed by cardiomyocytes, fibroblasts, smooth muscle cells and vascular endothelial wall cells [8]. Basic scientific research showed that PAR-1 may stimulate pathological remodeling after cardiac ischemia/reperfusion injury [8,9]. Moreover, PAR-1 is involved in hemostasis and thrombosis [8,10,11]. PAR-1 modulates thrombin signaling and is expressed on platelets, and may activate platelet secretion and aggregation. Local tissue injury of the vascular endothelial wall might induce endothelial responses via PAR-1, like recruitment of leukocytes and platelets, to manage infection or damage [11]. Accordingly, higher PAR-1 serum levels during the first 30 days following an ACS, might play a role in pathological remodeling after an ACS, and may lead to the development of (platelet-dependent) arterial thrombosis, and thus the recurrence of a coronary event.

BMP-6 is part of the transforming growth factor β family of cytokines. BMP-6 is involved in activation of osteogenic markers in mesenchymal stem cells, and may modulate ectopic cartilage and bone matrix formation [12]. Bone matrix formation is one of the key processes responsible for vascular calcification [13]. Since BMPs are overexpressed in (vulnerable) atherosclerotic lesions, it is suggested that BMPs modulate vascular calcification [14]. Furthermore, it is observed that BMPs contribute to vascular inflammation [14-16]. Lastly, previous research has indicated that oxidative stress may induce BMP-6 expression and thereby vascular inflammation and calcification [12]. Thus, it could be hypothesized that post-ACS oxidative stress may induce higher BMP-6 serum levels, which - in turn - might induce vascular inflammation and a recurrent coronary event.

ANG-1 is a widely expressed biomarker and is involved in multiple cellular processes

that occur following an ACS [17-19]. ANG-1 modulates endothelial cell survival, proliferation, migration and reorganization. Furthermore, it promotes angiogenesis and vascular quiescence. However, in the absence of vascular endothelial growth factor (VEGF) exposure, ANG-1 may promote vessel regression [18].

To the contrast of our study, previous research indicated that ANG-1 positively modulates cardiovascular disease, and promotes cardiomyocyte survival and reduces infarct size [20-22]. Furthermore, one study showed that a lower serum level of ANG-1 on admission date, significantly predicted the development of one-year major cardiovascular events in post-ACS patients [21].

Despite the complexity of the process, it is of interest to study blood biomarkers that stimulate the intracellular MAPK-cascade, since they may serve as novel biomarkers for aggravation of CVD. Because of the exploratory character of this study, our study results are limited to the examination of divergent biomarker patterns and thus, primarily, are valuable for exploration and identification of (novel) protein blood biomarkers. Further research is needed to establish whether the studied protein blood biomarkers may actually be used to identify post-ACS patients who are at higher risk of developing a recurrent coronary event.

Limitations

Since we chose to use Olinks' Proteomics PEA high throughput analysis to efficiently analyze our samples for potential discovery of novel protein blood biomarkers, our study results lack generalizability because of the use of arbitrary units. In addition, although Olinks' Proteomics PEA is an assay that gives highly reproducible results [23]. PEA technique still needs improvements to assure complete reproducibility. Lastly, because of the small number of events in our study, differences between cases and controls may have been masked.

Conclusion

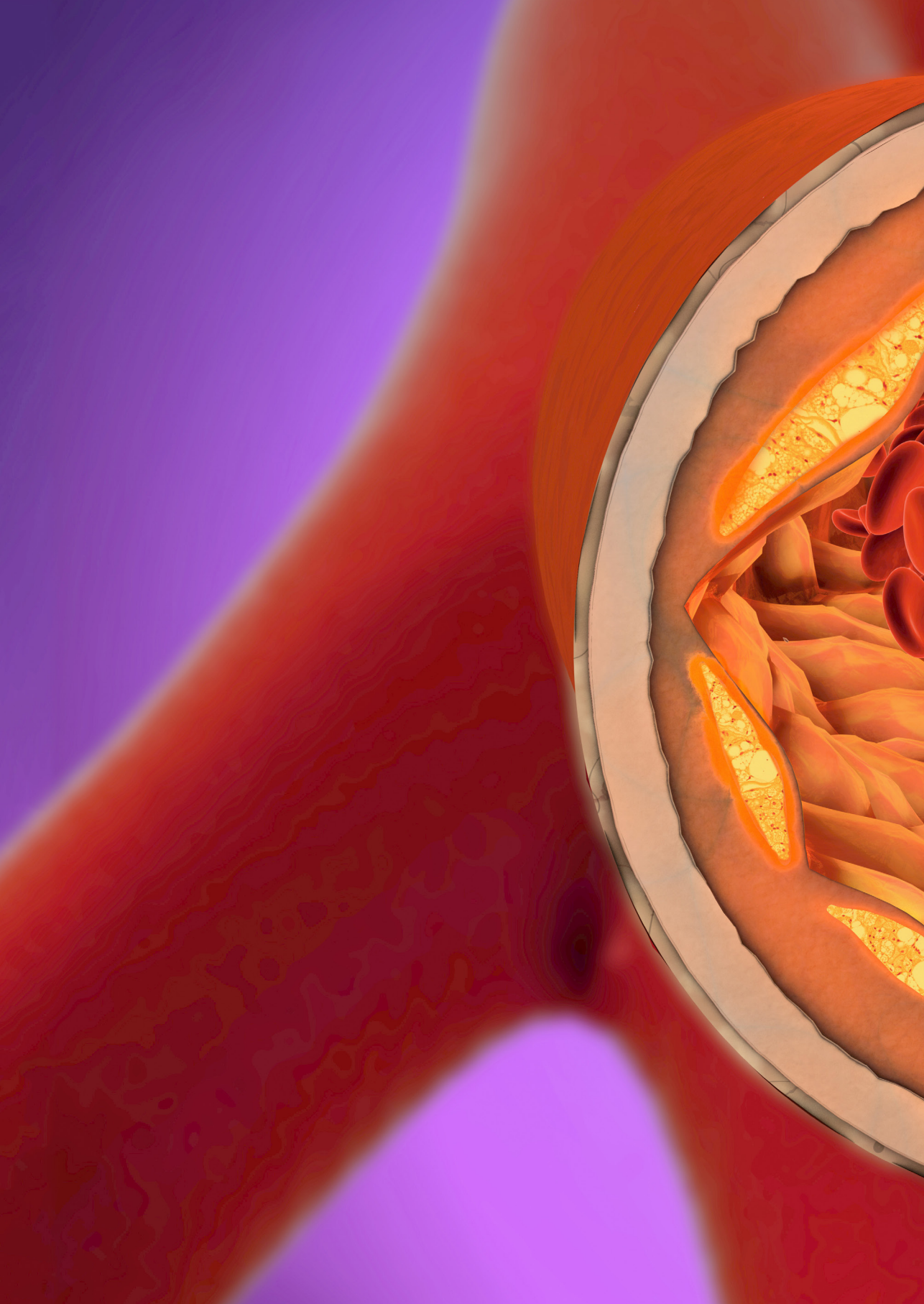
In conclusion, the serum levels of ANG-1, PAR-1 and BMP-6, all biomarkers that stimulate the MAPK-cascade, were significantly elevated in patients with ACS who developed an early recurrent coronary event. These signaling proteins warrant further study on their potential use as novel biomarkers to identify high risk post-ACS patients.

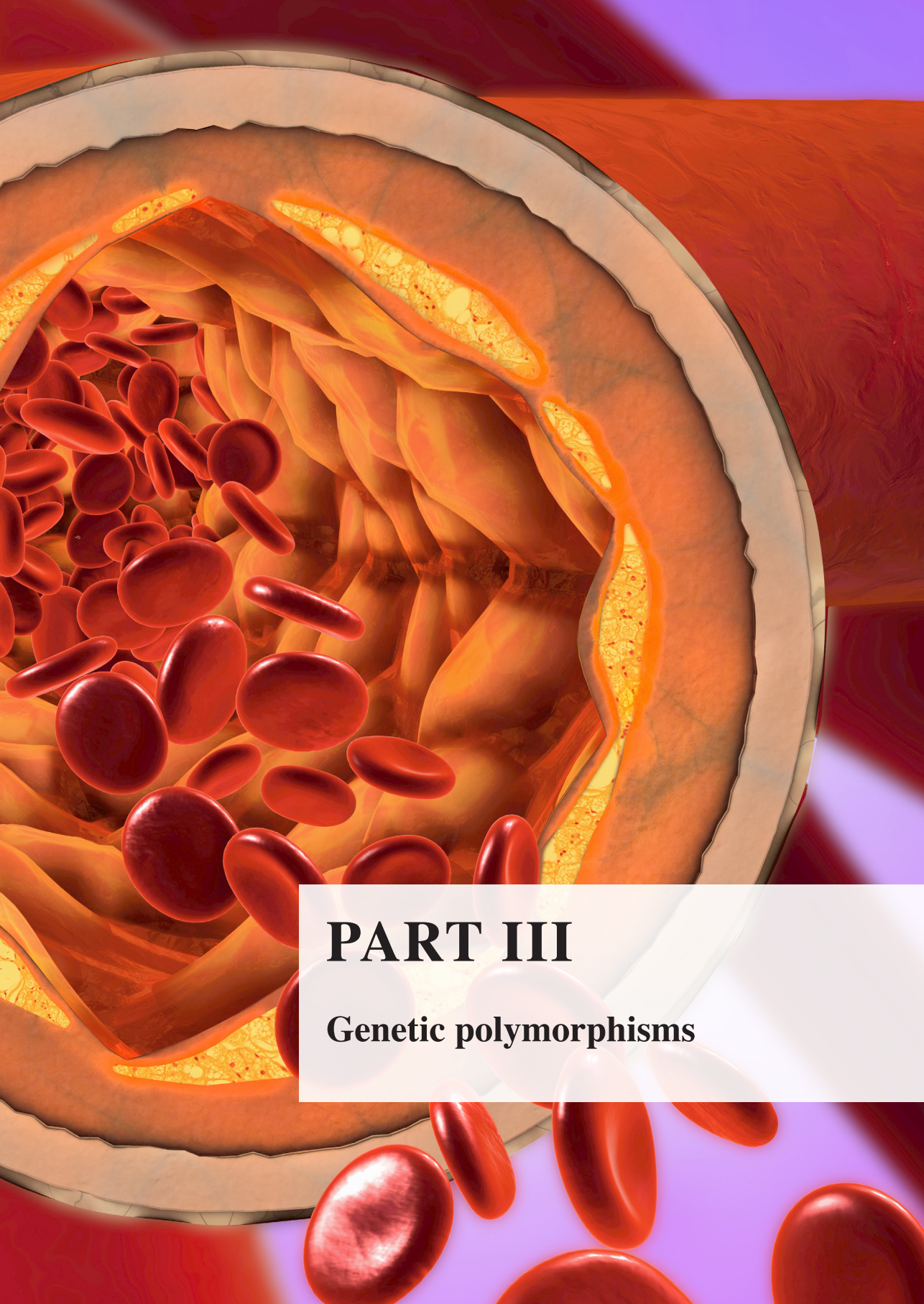
References

1. Nauta S.T. et al. Changes in clinical profile, treatment, and mortality in patients hospitalised for acute myocardial infarction between 1985 and 2008. PLoS One 2011; 6: e26917.
2. Townsend N. et al. Cardiovascular disease in Europe: epidemiological update 2016. Eur Heart J 2016; 37: 3232-3245.
3. van den Berge J.C. et al. Temporal trends in long-term mortality of patients with acute heart failure: Data from 1985-2008. Int J Cardiol 2016; 224: 456-460.
4. Raman M. et al. Differential regulation and properties of MAPKs. Oncogene 2007; 26: 3100-3112.
5. Muslin A.J. MAPK signalling in cardiovascular health and disease: molecular mechanisms and therapeutic targets. Clin Sci (Lond) 2008; 115: 203-218.
6. Oemrawsingh R.M. et al. Cohort profile of BIOMArCS: the BIOMarker study to identify the Acute risk of a Coronary Syndrome-a prospective multicenter biomarker study conducted in the Netherlands. BMJ Open 2016; 6: e012929.
7. Li J. and Ji L. Adjusting multiple testing in multilocus analyses using the eigenvalues of a correlation matrix. Heredity (Edinb) 2005; 95: 221-227.
8. Antoniak S. et al. Protease-activated receptors and myocardial infarction. IUBMB Life 2011; 63: 383-389.
9. Pawlinski R. et al. Protease-activated receptor-1 contributes to cardiac remodeling and hypertrophy. Circulation 2007; 116: 2298-2306.
10. Coughlin S.R. Thrombin signalling and protease-activated receptors. Nature 2000; 407: 258-264.
11. Kahn M.L. et al. Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. J Clin Invest 1999; 103: 879-887.
12. Yung L.M. et al. Bone morphogenetic protein 6 and oxidized low-density lipoprotein synergistically recruit osteogenic differentiation in endothelial cells. Cardiovasc Res 2015; 108: 278-287.
13. Parhami F. et al. High-density lipoprotein regulates calcification of vascular cells. Circ Res 2002; 91: 570-576.
14. Bostrom K. et al. Bone morphogenetic protein expression in human atherosclerotic lesions. J Clin Invest 1993; 91: 1800-1809.
15. Derwall M. et al. Inhibition of bone morphogenetic protein signaling reduces vascular calcification and atherosclerosis. Arterioscler Thromb Vasc Biol 2012; 32: 613-622.
16. Yao Y. et al. Inhibition of bone morphogenetic proteins protects against atherosclerosis and vascular calcification. Circ Res 2010; 107: 485-494.
17. Fukuhara S. et al. Differential function of Tie2 at cell-cell contacts and cell-substratum contacts regulated by angiopoietin-1. Nat Cell Biol 2008; 10: 513-526.
18. Maisonpierre P.C. et al. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 1997; 277: 55-60.
19. Saharinen P. et al. Angiopoietins assemble distinct Tie2 signalling complexes in endothelial cell-cell and cell-matrix contacts. Nat Cell Biol 2008; 10: 527-537.
20. Lee K.W. et al. Plasma angiopoietin-1, angiopoietin-2, angiopoietin receptor tie-2, and vascular endothelial growth factor levels in acute coronary syndromes. Circulation 2004; 110: 2355-2360.
21. Liu K.L. Plasma angiopoietin-1 level, left ventricular ejection fraction, and multivessel disease predict development of 1-year major adverse cardiovascular events in patients with acute ST elevation myocardial infarction - a pilot study. Int J Cardiol 2015; 182: 155-160.
22. Sun L. et al. Mesenchymal stem cells modified with angiopoietin-1 improve remodeling in a rat model of acute

myocardial infarction. Biochem Biophys Res Commun 2007; 357: 779-784.

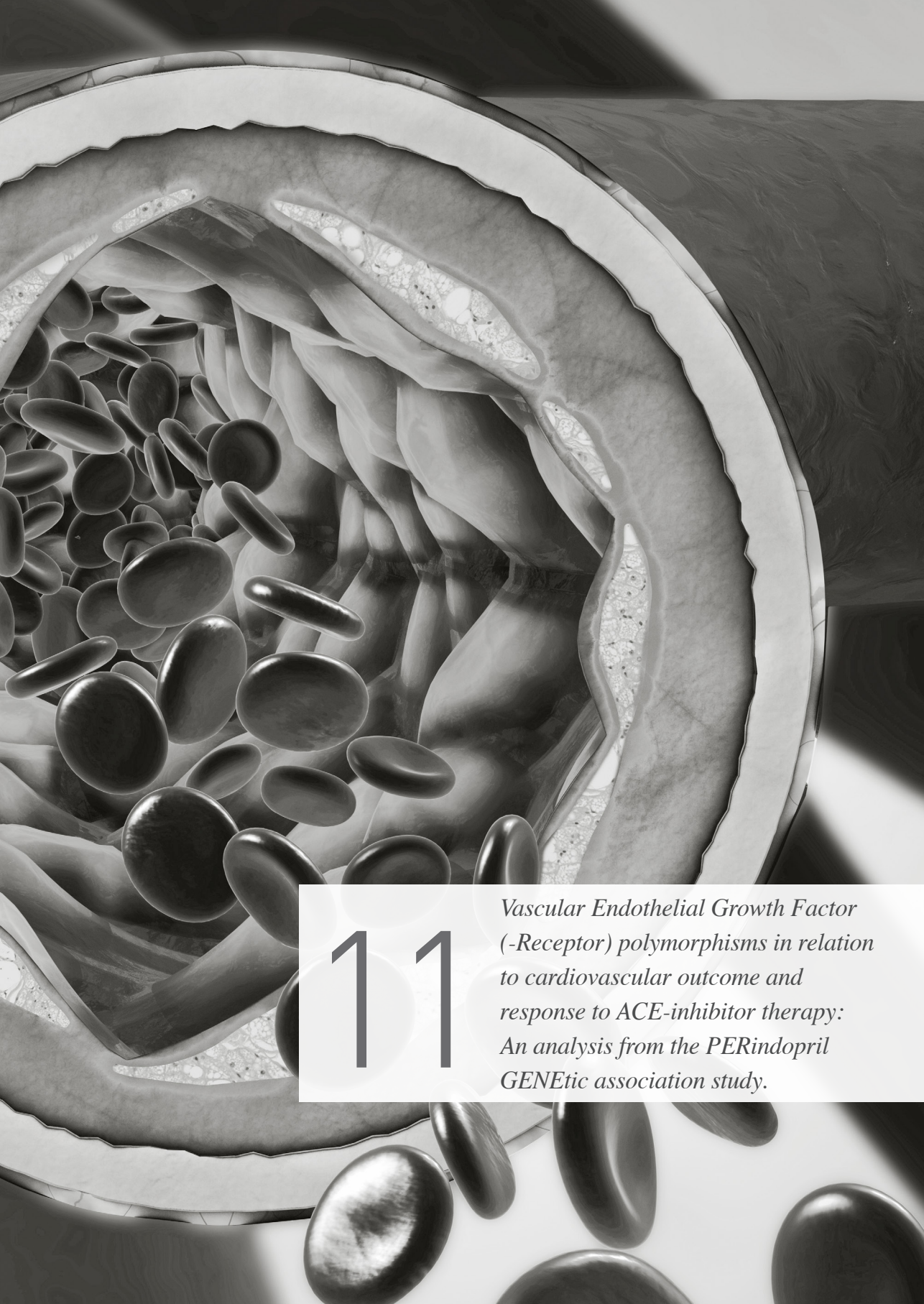
23. Assarsson E. et al. Homogenous 96-plex PEA immunoassay exhibiting high sensitivity, specificity, and excellent scalability. PLoS One 2014; 9: e95192.





PART III

Genetic polymorphisms



11

Vascular Endothelial Growth Factor (-Receptor) polymorphisms in relation to cardiovascular outcome and response to ACE-inhibitor therapy: An analysis from the PERindopril GENEtic association study.

11

Vascular Endothelial Growth Factor (-Receptor) polymorphisms in relation to cardiovascular outcome and response to ACE-inhibitor therapy: An analysis from the PERindopril GENEtic association study.

Buljubasic N, Kappers MHW, Dehghan A, Brugts JJ, Versmissen J, Remme WJ, Bertrand ME, Fox KM, Ferrari R, Simoons ML, Boersma E, Kardys I, Akkerhuis KM.

Submitted.

Abstract

Rationale: Variations in the vascular endothelial growth factor (VEGF) and kinase insert domain-containing receptor (KDR) genes affect gene expression or the function of the encoded proteins. Studies investigating VEGF/KDR polymorphisms in relation to cardiovascular outcome have been relatively small and yielded inconsistent results. We investigated the relationship between seven VEGF/KDR variants with cardiovascular outcome, as well as with the magnitude of the treatment benefit provided by the ACE-inhibitor perindopril.

Methods & Results: In 8711 stable coronary artery disease (CAD) patients, randomized to perindopril or placebo during a median follow-up of 4.3 years, four VEGF (rs699947, rs1570360, rs2010963, rs3025039) and three KDR (rs2071559, rs2305948, rs1870377) polymorphisms were determined. The primary endpoint was the composite of cardiovascular mortality, myocardial infarction, or cardiac arrest. Although no associations were present

between any of the individual polymorphisms or their haplotypes with cardiovascular events in multivariable analysis, three variants (rs699947, rs2010963, rs2305948) showed differential effects of perindopril on cardiovascular outcome. Of all patients, 50.2% experienced an enhanced treatment effect (HR 0.71; 95% CI 0.60-0.83), whereas 49.8% had no beneficial effect of perindopril (HR 1.13; 95% CI 0.83-1.55) on the primary endpoint (interaction p-value 0.012).

Conclusions: VEGF/KDR polymorphisms cannot be used for risk stratification in patients with stable CAD, since there was no association with cardiovascular outcome. However, multiple VEGF/KDR polymorphisms modified the treatment effect by perindopril on adverse cardiovascular outcome. VEGF/KDR polymorphisms might therefore be used for identification of patients with an incremental treatment benefit of perindopril.

Introduction

Coronary atherosclerosis is a multifactorial process, affected by genetic influences, eventually leading to coronary artery disease (CAD). Patients with stable CAD have an increased risk of developing adverse cardiovascular events [1]. To optimize the benefits of secondary prevention in these patients, various clinical tools have been evaluated to improve risk stratification [2]. In recent years, single nucleotide polymorphisms (SNPs) involved in coronary atherosclerosis have shown promise as a risk assessment tool to improve cardiovascular risk prediction in established CAD [3]. For example, variations in genes involved in inflammation, oxidative stress and lipid metabolism have been extensively investigated and identified to be associated with both coronary atherosclerosis and incident coronary events [4-6]. Investigation on functional polymorphisms in candidate genes regulating other biological processes in atherosclerosis may further increase our knowledge of genetic susceptibility to CAD and thereby contribute to the improvement of secondary prevention in stable CAD.

Vascular endothelial growth factor (VEGF) is a crucial mitogen in the vascular wall that exerts its effects mainly by binding to different receptors, most importantly the kinase insert domain-containing receptor (KDR) - also known as VEGF-receptor 2 - on the endothelium, which locally stimulates vascular endothelial cell proliferation and angiogenesis [7]. Experimental studies have suggested an important role for VEGF/KDR in atherosclerotic

plaque progression through its ability to enhance vascular permeability, modulate thrombogenicity and mediate inflammation, as well as neovascularization in atherosclerotic plaques [8]. Despite these deleterious effects on the vascular wall, VEGF/KDR has also been shown to exert beneficial effects on myocardial ischemia by promoting collateral blood supply [9]. Therefore, whether VEGF and its receptors have an overall protective or harmful effect in CAD remains unresolved.

The functional regions of the VEGF and KDR genes contain several SNPs that have been associated with different levels of expression of VEGF and KDR [10-14]. These SNPs may therefore be of influence on the risk of the development of CAD or its sequelae [15]. Until now, there are only a few studies that have investigated VEGF/KDR polymorphisms in relation to CAD. Moreover, these studies have been relatively small, and have yielded inconsistent results [13, 16-19].

Angiotensin-converting enzyme (ACE)-inhibitors have shown benefit in improving outcome in stable CAD. For optimal treatment benefit, it is pivotal to identify those patients who are most likely to gain from therapy. Genetic variants may be instrumental to target pharmacological treatment at an individual level and reach an optimal treatment response. Several polymorphisms in the renin-angiotensin aldosterone system (RAAS) and kallikrein-bradykinin (KB) pathway have been shown to modulate the treatment benefit of the ACE-inhibitor perindopril [20]. There are also indications that ACE-inhibitors might indirectly affect the VEGF/KDR pathway through activation of the bradykinin pathway (Figure 1), which has led to the hypothesis that variants in the VEGF/KDR genes may also modulate the treatment benefit of the ACE-inhibitors. This has not been investigated before.

Therefore, the purpose of the present study was twofold. First, in order to elucidate the role of VEGF/KDR polymorphisms in CAD, the present study evaluates the associations between the seven most common VEGF/KDR polymorphisms and their haplotypes with incident cardiovascular events in 8711 patients with stable CAD. Second, we assessed whether VEGF/KDR polymorphisms and their haplotypes modulate the treatment benefit of ACE-inhibitor therapy with perindopril in this patient population.

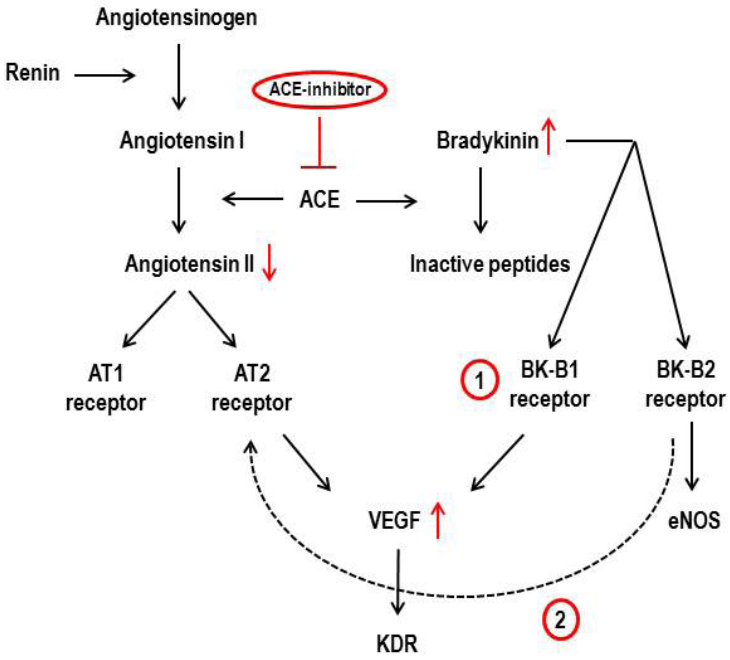


Figure 1. Simplified scheme of the renin-angiotensin aldosterone system and bradykinin pathway and its hypothesized interaction with VEGF.

ACE=angiotensin converting enzyme; AT1 receptor=angiotensin II type 1 receptor; AT2 receptor=angiotensin II type 2 receptor; BK-B1 receptor=bradykinin type B1 receptor; BK-B2 receptor=bradykinin type B2 receptor; eNOS=endothelial nitric oxide; KDR=kinase insert domain-containing receptor; VEGF=vascular endothelial growth factor. Two proposed mechanisms of the effects of ACE-inhibitors on VEGF. It is hypothesized that VEGF is upregulated by ACE-inhibitors through activation of the bradykinin pathway. On the one hand, the bradykinin B1 receptor stimulates VEGF expression directly (1). On the other hand, a possible interaction between bradykinin B2 receptor and angiotensin II type 2 receptor may also result in enhanced VEGF expression (2).

Methods

Study design and population

The current investigation was embedded in the PERindopril GENEtic Association (PERGENE) study, a substudy of the EURopean trial On reduction of cardiac events with Perindopril in stable coronary Artery disease (EUROPA). These studies have been described in detail previously [21,22].

In brief, the EUROPA trial was a randomized, double-blind, placebo-controlled clinical trial designed to assess the effect of the ACE-inhibitor perindopril on adverse cardiovascular outcome in 12.218 patients with stable CAD. The participants, recruited

between 1997 and 2000, had clinical evidence of CAD (documented previous myocardial infarction (MI), percutaneous or surgical coronary resvascularization, angiographic evidence of ≥70% narrowing of one or more major coronary arteries, or a history of typical chest pain in male patients with an abnormal stress test), but without heart failure, uncontrolled hypertension or recent use of ACE-inhibitors / angiotensin-receptor blockers. After a 4-week run-in period, in which all patients received perindopril once daily in addition to their normal medication, the participants were randomly assigned to either perindopril once daily or a matching placebo. PERGENE was a cardiovascular pharmacogenetic substudy (n=8746) that successfully identified three polymorphisms in the RAAS and bradykinin pathway, that significantly modified the treatment benefit of the ACE-inhibitor perindopril on adverse cardiovascular outcome [20,21,23]. The studies were approved by the institutional review board of every participating center and written informed consent was obtained from all patients for performing genetic analyses.

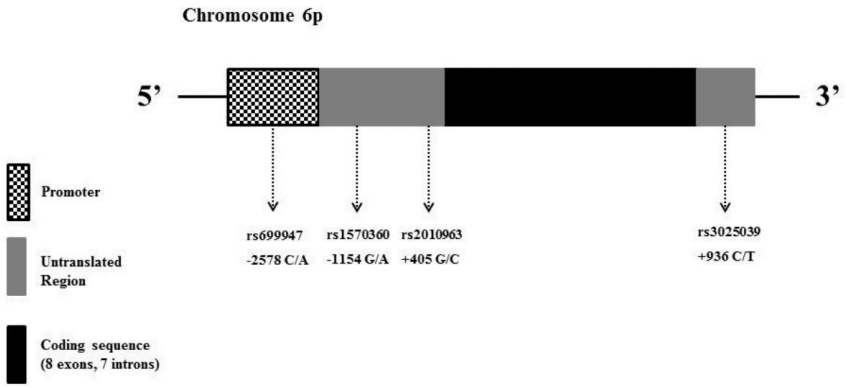
Study endpoints

The primary endpoint was a composite of cardiovascular mortality, non-fatal MI, and cardiac arrest with successful resuscitation. Secondary endpoints included cardiovascular mortality, MI (fatal and non-fatal), the composite of cardiovascular mortality and non-fatal MI, and the composite of cardiovascular mortality, non-fatal MI and revascularization (coronary artery bypass graft or percutaneous coronary intervention). An independent clinical event committee adjudicated all events using source documentation.

SNPs

All participants were genotyped for the rs699947 (-2578 C/A), rs1570360 (-1154 G/A), rs2010963 (405 G/C or -634 G/C), and rs3025039 (936 C/T) SNPs of the VEGF gene and rs2071559 (-604C/T), rs2305948 (1192 G/A), and rs1870377 (1719 A/T) SNPs of the KDR gene (Figure 2A and 2B). These seven SNPs were selected from existing literature based on their functional properties in atherosclerosis and CAD [18, 24-27]. Exact details on blood sample logistics, DNA isolation and genotyping techniques have been described previously [21]. In brief, after protocolized large-scale blood sample collection and storage, DNA from 8746 patients was successfully isolated using an automated isolation process (Hamilton liquid handler coupled with Magnetic separator for automated DNA extraction, Nevada, USA). Genotypes were determined with the Taqman allelic discrimination assay (Applied Biosystems,

Foster City, California, USA) by the ABI Taqman 7900HT. For the current investigation, only patients with complete data on follow-up, covariates and who were successfully genotyped were selected from the PERGENE substudy for further analysis (n=8711).

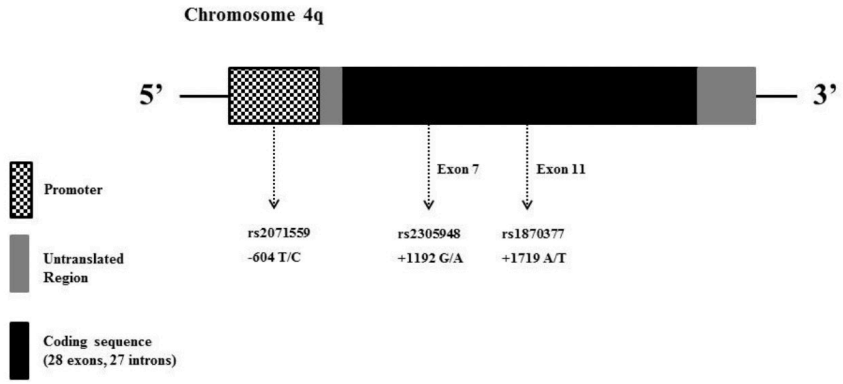


Haplotype	rs699947	rs1570360	rs2010963	Frequency (full cohort)
1	A*	A*	G	32.04%
2	C	G	C*	31.98%
3	C	G	G	18.28%
4	A*	G	G	16.93%
Others	-	-	-	≤ 0.33%

* Minor allele from that SNP used for that haplotype

Figure 2A. The VEGF protein gene (on chromosome 6p), its corresponding SNPs as determined in this study and common VEGF protein gene haplotypes.

Three out of the four VEGF SNPs (rs699947, rs1570360 and rs2010963) were in strong linkage disequilibrium (D' >0.97), and therefore selected for haplotype reconstruction. Ultimately, four haplotypes with a frequency of >0.3% were reconstructed.



Haplotype	rs2305948	rs1870377	Frequency (full cohort)
1	G	T*	70.23%
2	G	A	19.48%
3	A*	A	6.55%
4	A*	T*	3.74%

* Minor allele from that SNP used for that haplotype

Figure 2B. The VEGF receptor / KDR gene (on chromosome 4q), its corresponding SNPs as determined in this study and common VEGF receptor / KDR gene haplotypes.

Two out of the three KDR SNPs (rs2305948 and rs1870377) were in strong linkage disequilibrium (D' >50), and therefore selected for haplotype reconstruction. Ultimately, four haplotypes with a frequency of >0.3% were reconstructed.

Statistical analysis

The observed genotype frequency distributions of the SNPs were tested for Hardy-Weinberg equilibrium using a Chi-square test. Cox proportional hazards regression analysis was used to examine the associations between genotypes and study endpoints. Univariable and multivariable analyses were performed, including all covariables that were related to the incidence of the primary endpoint in the EUROPA study. These included age, gender, hypertension, diabetes mellitus, smoking (in the last month), hypercholesterolemia, body mass index >30, previous MI, previous stroke or peripheral vascular disease, symptomatic CAD and family history of CAD. We assumed an additive genotype model and accordingly, the homozygous common allele genotype was used as the reference category for each SNP. Genotypes were entered as 0 (homozygous common allele), 1 (heterozygous) and 2

(homozygous minor allele).

Subsequently, treatment effect modification of perindopril by genotype was examined. First, hazard ratios for the endpoints associated with perindopril use were calculated for each genotype of the SNPs to quantify the treatment effect. Treatment effect was defined as the reduction in the event rate of the cardiovascular endpoints (perindopril versus placebo). Afterwards, interaction terms (genotype x randomly allocated treatment) were included in the multivariable models to evaluate modification of treatment effect by genotype on outcome (P-value for interaction).

Based on separate SNP analyses for treatment effect modification as described above, a pharmacogenetic score was constructed. Eventually, in our analysis this score encompassed alleles from the 3 SNPs (rs699947, rs2010963 and rs2305948) that modified the treatment effect. By counting the number of alleles that were associated with a decreased benefit of treatment with perindopril, score categories ranging from 0 to 6 were formed. Hence, an increasing pharmacogenetic score indicated more unfavourable alleles. For each category of the score, the treatment effect of perindopril was calculated on primary and secondary outcome using a multivariable Cox proportional hazards regression analysis. Based on additional analyses and for practical purposes, patients were divided in groups with score <4, equal to 4 and >4.

Haploview 4.2 was used to examine linkage patterns between the SNPs in order to reconstruct haplotypes, which are specific combinations of multiple jointly inherited adjacent SNPs on the same chromosome [28]. Three out of the four VEGF SNPs (rs699947, rs1570360 and rs2010963) were in strong linkage disequilibrium (D' >0.97), and therefore selected for haplotype reconstruction. Similarly, two out of the three KDR SNPs (rs2305948 and rs1870377; D' >50) were selected for haplotype reconstruction. Haplotype reconstruction and haplotype frequency estimation were carried out with the expectation-maximization (EM) algorithm implemented in the Haplo Stats R package, version 1.7.7 (https://cran.r-project.org/web/packages/haplo.stats/haplo.stats.pdf). Briefly, this software contains a probabilistic statistical framework that uses maximum likelihood methods and iteratively estimates haplotype probabilities for each haplotype pair per individual based on the observed genotypes [29]. Herewith, all possible combinations of haplotype pairs within an individual are properly weighted rather than underestimating the variance by assigning a most likely pair of haplotype to an individual. Ultimately, a total of eight VEGF/KDR haplotypes with a frequency of >0.3% were reconstructed. Then, to estimate the magnitude of the effect

of each haplotype on cardiovascular outcome, a probability-weighted regression analysis was applied using the haplo.glm function of Haplo Stats. Effects were expressed by odds ratios with 95% confidence intervals. The most frequently observed haplotype served as the reference category. Multivariable analysis was performed and contained adjustments for the variables as described above.

P-values <0.05 were considered statistically significant. Statistical analyses were performed using the Statistical Package for Social Science (IBM SPSS statistics Inc., Chicago, IL, USA) for Windows, version 21.0. Haplotype analyses were performed with RStudio (RStudio: Integrated Development for R, RStudio Inc., Boston, MA, USA) for Windows, version 1.0.136.

Results

Baseline characteristics of the study patients (n=8711) are presented in Table 1. Mean age was 59.8±9.3 years and 85.5% were men. At baseline, mean systolic and diastolic blood pressure were 136.9±15.2 mmHg and 81.8±8.1 mmHg, respectively. The genotype distributions of the VEGF/KDR polymorphisms were in Hardy-Weinberg equilibrium (Table 2). The exact locations of the seven SNPs and frequencies of the haplotypes used for analysis are depicted in Figure 2A and 2B.

Genotype in relation to cardiovascular outcome

The primary endpoint occurred in 793 (9.1%) patients during a follow-up of 4.3 [4.0 – 4.5] (median [interquartile range]) years (Supplemental Table 1). The primary endpoint, as well as all secondary endpoints, occurred less frequently in patients receiving the ACE-inhibitor perindopril as compared to those receiving placebo. There was no significant association between any of the individual VEGF/KDR SNPs and the primary (Table 3) or secondary endpoints (Supplemental Table 2) in multivariable analysis. There was also no significant association between any of the VEGF/KDR haplotypes and cardiovascular outcome in multivariable analysis (Supplemental Table 3A and 3B).

Table 1. Baseline patient characteristics (n=8711).

	Total study population	Pharmacogenetic score ≤ 4	Pharmacogenetic score > 4
Patient characteristics			
Age, years	59.8 ± 9.3	59.8 ± 9.2	60.0 ± 9.5
Male gender, n (%)	7450 (85.5)	5839 (85.6)	1426 (85.4)
Cardiovascular risk factors, n (%)			
Hypertension	2485 (28.5)	1965 (28.8)	460 (27.6)
Diabetes mellitus	1106 (12.7)	845 (12.4)	226 (13.5)
Hypercholesterolemia	5480 (62.9)	4259 (62.4)	1079 (64.6)
Smoking (in the last month)	1285 (14.8)	1018 (14.9)	243 (14.6)
Body mass index > 30kg/m^2	1857 (21.3)	1437 (21.1)	379 (22.7)
Family history of coronary artery disease	2371 (27.2)	1836 (26.9)	466 (27.9)
Medical history, n (%)			
History of coronary artery disease			
Myocardial infarction	5689 (65.3)	4446 (65.2)	1091 (65.4)
Revascularization	4763 (54.7)	3758 (55.1)	881 (52.8)
History of peripheral vascular disease	646 (7.4)	500 (7.3)	126 (7.5)
History of stroke or TIA	310 (3.6)	247 (3.6)	54 (3.2)
Medication use, n (%)			
Platelet inhibitors	8031 (92.2)	6290 (92.2)	1537 (92.1)
Oral anticoagulants	378 (4.3)	295 (4.3)	72 (4.3)
β blockers	5507 (63.2)	4312 (63.2)	1060 (63.5)
Lipid-lowering agents	4818 (55.3)	3780 (55.4)	918 (55.0)
Clinical characteristics, n (%)			
Randomised to treatment group			
Perindopril (ACE-inhibitor)	4329 (49.7)	3383 (49.6)	828 (49.6)
Placebo	4382 (50.3)	3438 (50.4)	841 (50.4)
Baseline blood pressure[a]			
Systolic blood pressure, mmHg	136.9 ± 15.2	136.9 ± 15.2	136.9 ± 15.4
Diastolic blood pressure, mmHg	81.8 ± 8.1	81.8 ± 8.1	81.8 ± 8.1
Blood pressure reduction by perindopril, mmHg[b]			
Systolic blood pressure, mmHg	8.6 ± 14.6	8.6 ± 14.5	8.7 ± 14.7
Diastolic blood pressure, mmHg	4.0 ± 8.5	4.0 ± 8.5	4.1 ± 8.6

TIA=Transient Ischemic Attack. Continuous variables are presented as mean ± standard deviation and categorical variables as percentages. [a]Baseline blood pressure measured at first screening visit, before the 4-week run-in period of the EUROPA-trial in which all patients were treated with perindopril. [b]Blood pressure reduction calculated as the mean difference in blood pressure from the first screening visit to randomization after the 4-week run-in period.

P-values for differences between patients with pharmacogenetic scores ≤4 and >4 were obtained using the independent Student's t-test for continuous variables and the χ^2 test for categorical variables. All p-values were non-significant (>0.05).

Table 2. VEGF and KDR protein SNP characteristics and distribution (n=8711).

	SNP		Location	Genotype, n (%)			HWE p-value
	Description	RS number		Homozygous common allele	Heterozygous	Homozygous minor allele	
VEGF	-2578 C / a	rs699947	Promoter	2183 (25.1)	4337 (49.8)	2076 (23.8)	0.93
	-1154 G / a	rs1570360	5' UTR	3976 (45.6)	3693 (42.4)	956 (11.0)	0.81
	+405 G / c[a]	rs2010963	5' UTR	3971 (45.6)	3738 (42.9)	929 (10.7)	0.91
	+936 C / t	rs3025039	3' UTR	6301 (72.3)	2172 (24.9)	187 (2.1)	0.98
KDR	-604 T / c	rs2071559	Promoter	2284 (26.2)	4276 (49.1)	2075 (23.8)	0.93
	+1192 G / a	rs2305948	Exon 7	6970 (80.0)	1605 (18.4)	90 (1.0)	0.96
	+1719 A / t	rs1870377	Exon 11	569 (6.5)	3356 (38.5)	4708 (54.0)	0.92

HWE=Hardy Weinberg Equilibrium; KDR=Kinase insert Domain-containing Receptor; SNP=Single Nucleotide Polymorphism; UTR=Untranslated Region; VEGF=Vascular Endothelial Growth Factor. [a]Also described as -634 G / c in previous literature. Categorical variables are presented as percentages. P-values for testing HWE were obtained using the χ^2 test.

Table 3. VEGF and KDR polymorphisms in relation to the primary endpoint (n=8711).

	SNP		HR (95% CI)	P-value
	RS number	Genotype		
VEGF	rs699947	CC	Reference	
		Ca	0.90 (0.76 - 1.07)	0.23
		aa	0.98 (0.80 - 1.19)	0.83
	rs1570360	GG	Reference	
		Ga	0.95 (0.82 - 1.10)	0.48
		aa	0.88 (0.69 - 1.11)	0.28
	rs2010963	GG	Reference	
		Gc	0.99 (0.85 - 1.15)	0.90
		cc	1.00 (0.79 - 1.27)	1.00
	rs3025039	CC	Reference	
		Ct	1.15 (0.98 - 1.34)	0.09
		tt	0.93 (0.57 - 1.53)	0.78
KDR	rs2071559	TT	Reference	
		Tc	1.01 (0.85 - 1.19)	0.92
		cc	0.97 (0.79 - 1.18)	0.75
	rs2305948	GG	Reference	
		Ga	0.97 (0.81 - 1.16)	0.71
		aa	0.86 (0.43 - 1.73)	0.67
	rs1870377	AA	Reference	
		At	1.04 (0.77 - 1.40)	0.80
		tt	1.03 (0.77 - 1.37)	0.87

CI=Confidence Interval; HR=Hazard Ratio; KDR=Kinase insert Domain-containing Receptor; SNP=Single Nucleotide Polymorphism; VEGF=Vascular Endothelial Growth Factor. Cox proportional hazard regression analysis was performed to estimate hazard ratios (95% confidence interval) for the primary endpoint, adjusted for the covariables mentioned in the method section. The homozygous common allele genotype of each SNP was used as the reference category.

Genotype in relation to treatment benefit of perindopril

Across the different genotypes of the VEGF gene (Supplemental Figure 1A-D), two out of the four SNPs showed a statistically significant differential effect of perindopril on several outcomes. Specifically, perindopril provided an incremental treatment benefit on several endpoints in minor allele carriers of rs699947 ('Ca' or 'aa') and common allele carriers of rs2010963 ('GG' or 'Gc') (Supplemental Figure 1A and 1C). For the other two SNPs, tendencies towards treatment effect modification were observed without the interaction terms reaching statistical significance (Supplemental Figure 1B and 1D).

Across the different genotypes of the KDR gene (Supplemental Figure 1E-G), out of the three SNPs, tendencies towards a differential treatment benefit of perindopril were observed only for rs2305948. Minor allele carriers of rs2305948 ('Ga' or 'aa') showed larger treatment benefit as compared to the homozygous common allele genotype ('GG') on the primary endpoint (Supplemental Figure 1F). Because of limited statistical power with less than 6 primary endpoint events in the 'aa' genotype, an incremental treatment benefit could not be statistically demonstrated in this group. In the other two investigated SNPs of the KDR gene, no treatment effect modification was seen (Supplemental Figure 1E and 1G).

The results of the VEGF/KDR haplotype analyses on treatment effect modification were comparable to the results of the individual SNP analyses (Supplemental Table 4A and 4B). The haplotype analysis identified similar alleles to be associated with either a decreased (haplotype C-G-c of the VEGF gene; Supplemental Table 4A) or an increased (haplotype a-t of the KDR gene; Supplemental Table 4B) treatment benefit on several endpoints. All aforementioned associations were independent of differences in blood pressure between perindopril- and placebo-treated patients, since additional analysis (results not shown) showed no differential effects of perindopril across different genotypes on blood pressure.

Pharmacogenetic score in relation to treatment benefit of perindopril

Based upon the separately analyzed SNPs, a pharmacogenetic score (composed of rs699947, rs2010963 and rs2305948) was constructed. With an increasing score the event rate of the primary endpoint increased in patients allocated to perindopril (from 3.5% to 5.0%) and decreased in patients allocated to placebo (from 5.4% to 4.5%) (Figure 3, Table 4). Thus, an increasing score containing more unfavourable alleles showed a decrease in treatment benefit of perindopril on the primary outcome. The group with score <4 experienced an increased treatment benefit (HR 0.64; 95% CI 0.53–0.80), whereas the group with score 4 or score >4

did not experience a treatment benefit (p-value of interaction=0.007) as depicted in Figure 3. Taken together, we identified 50.2% of the total study population with an improved treatment effect (score ≤4; HR 0.71; 95% CI 0.60–0.83) and 49.8% of the population without benefit from treatment with perindopril (score >4; HR 1.13; 95% CI 0.83–1.55) on the primary endpoint. The p-value of interaction between treatment effect and risk score ≤4 or >4 was 0.012. Patients allocated to placebo with score >4 had a reduced risk (HR 0.86; 95% CI 0.67–1.10) as compared to those with a score ≤4. In contrast, patients with a score >4 within the perindopril group had a significantly higher risk (HR 1.35; 95% CI 1.05–1.73) than those with a score ≤4, demonstrating the interaction of the pharmacogenetic score and treatment benefit. The observed treatment interaction effect cannot be explained by clinical differences, since no differences in clinical characteristics existed between patients with score >4 and ≤4 (Table 1).

It is important to note that all above mentioned results and observed trends are also applicable to the secondary cardiovascular endpoints we examined (Table 4).

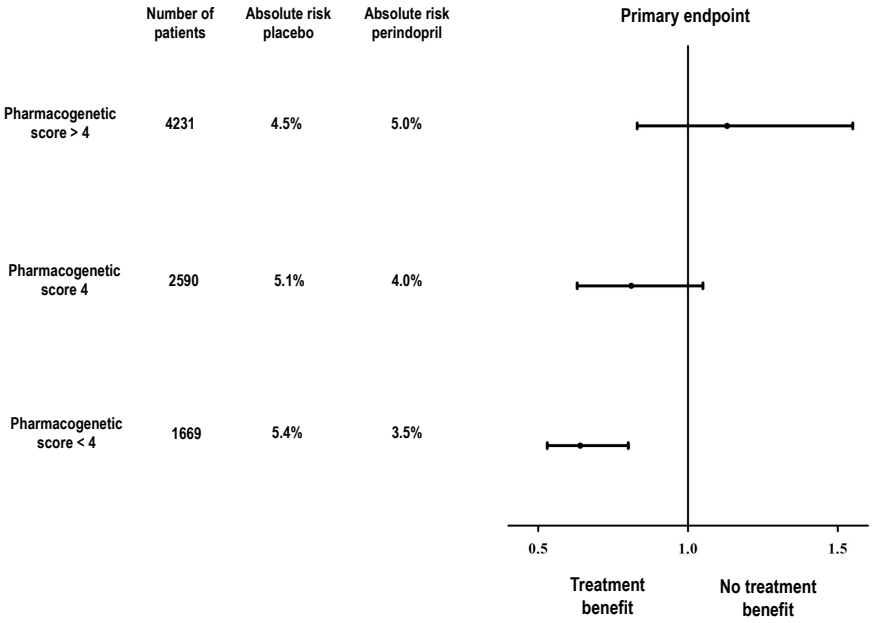


Figure 3. Pharmacogenetic score and the treatment effect of perindopril on the primary endpoint.

Multivariable Cox proportional hazard regression analysis applied with adjustment for the covariables mentioned in the method section. P-value of interaction between pharmacogenetic score and treatment effect was 0.007.

Table 4. Pharmacogenetic score and the treatment effect of perindopril (n=8490).

	Pharmacogenetic score	Events, n (%)		HR (95% CI)	P-value for interaction
		Perindopril group	Placebo group		
Cardiovascular mortality, non-fatal myocardial infarction and cardiac arrest (primary endpoint)	< 4	146 (3.5)	227 (5.4)	0.64 (0.53 – 0.80)	0.007
	4	103 (4.0)	133 (5.1)	0.81 (0.63 – 1.05)	
	> 4	83 (5.0)	75 (4.5)	1.13 (0.83 – 1.55)	
Cardiovascular mortality	< 4	71 (1.7)	97 (2.3)	0.74 (0.55 – 1.01)	0.19
	4	44 (1.7)	53 (2.0)	0.91 (0.61 – 1.36)	
	> 4	39 (2.3)	33 (2.0)	1.19 (0.75 – 1.89)	
Non-fatal myocardial infarction	< 4	81 (1.9)	144 (3.4)	0.57 (0.43 – 0.75)	0.010
	4	62 (2.4)	83 (3.2)	0.76 (0.55 – 1.06)	
	> 4	47 (2.8)	47 (2.8)	1.02 (0.68 – 1.53)	
Non-fatal or fatal myocardial infarction	< 4	92 (2.2)	162 (3.8)	0.58 (0.45 – 0.75)	0.017
	4	65 (2.5)	88 (3.4)	0.76 (0.55 – 1.04)	
	> 4	51 (3.1)	54 (3.2)	0.96 (0.65 – 1.41)	
Cardiovascular mortality or non-fatal myocardial infarction	< 4	144 (3.4)	225 (5.3)	0.65 (0.52 – 0.80)	0.006
	4	102 (3.9)	130 (5.0)	0.82 (0.63 – 1.07)	
	> 4	82 (4.9)	75 (4.5)	1.12 (0.82 – 1.53)	
Cardiovascular mortality, non-fatal myocardial infarction or revascularization (CABG or PCI)	< 4	278 (6.6)	371 (8.8)	0.64 (0.52 – 0.79)	0.07
	4	189 (7.3)	220 (8.5)	0.82 (0.64 – 1.07)	
	> 4	133 (8.0)	137 (8.2)	1.12 (0.82 – 1.54)	

CABG=Coronary Artery Bypass Graft surgery; CI=Confidence Interval; HR=Hazard Ratio; PCI=percutaneous coronary intervention. Pharmacogenetic score, composed of 3 single nucleotide polymorphisms (rs699947, rs2010963, rs2305948), and the treatment effect of perindopril in stable coronary artery disease patients. In total, 8490 out of 8711 patients had complete genotype data on rs699947, rs2010963 and rs2305948. The groups with score <4, 4 and >4 consisted of respectively 4231 (49.8%), 2590 (30.5%) and 1669 (19.7%) patients. Cox proportional hazard regression analysis was performed to estimate hazard ratios (95% confidence interval) for the primary and secondary endpoints, adjusted for the covariables mentioned in the method section. The p-value for interaction between pharmacogenetic score and treatment effect was calculated by adding an interaction term to the model.

Discussion

The present study is the first large clinical study that has comprehensively investigated the associations of seven VEGF/KDR polymorphisms and their haplotypes with cardiovascular outcome, as well as with effect modification of the ACE-inhibitor (perindopril)-induced treatment benefit, in patients with stable CAD. We did not find any associations between individual VEGF/KDR polymorphisms or their haplotypes with 4-year cardiovascular outcome. However, multiple VEGF/KDR variants, especially when combined into a proposed pharmacogenetic score, showed modification of the treatment effect of perindopril on cardiovascular outcome. Altogether, these findings do not support that VEGF/KDR polymorphisms are useful for risk assessment in patients with stable CAD, but suggest that

they might rather be used to increase treatment efficacy of the ACE-inhibitor perindopril with regard to prevention of coronary events.

Individual SNPs and cardiovascular outcome

VEGF protein is a known mitogen and potent angiogenic factor, expressed mostly by endothelial cells, that binds to the tyrosine kinase receptor on endothelial cells and through which VEGF exerts its many (patho)physiological effects [7]. The Kinase insert Domain-containing Receptor (KDR), also known as VEGF receptor 2 or Fetal Liver Kinase 1, is generally recognized to have a predominant role in mediating a variety of VEGF-induced responses on the vascular wall [7]. Due to pro-angiogenic and pro-inflammatory properties of the VEGF/KDR pathway, it is hypothesized to promote atherosclerosis. Previous studies have shown that serum VEGF concentrations are associated with atherosclerosis and are elevated in CAD [30,31]. Moreover, immunohistochemical studies have shown the presence of VEGF in human coronary atherosclerotic segments and the absence of VEGF expression in normal coronary arteries [32]. These findings indicate that VEGF may have a role in the progression of human coronary atherosclerosis and in the development of CAD. It has been well established that heritability accounts for 80% of the total variation of circulating levels of VEGF [33]. In other words, genetic variants in the VEGF and KDR genes primarily determine alterations in circulating VEGF levels and KDR functionality. Transcriptional regulation of VEGF/KDR gene expression could be possibly altered by certain genetic variances, leading to different circulating protein levels [14,34]. The studied polymorphisms in the present investigation are known to be correlated with VEGF production and KDR biological functional activity.

A few case-control studies have previously investigated the relationship between VEGF/KDR polymorphisms and the prevalence of CAD, yet their results have been inconclusive [13,16,17,19]. Thus, it has remained unclear whether VEGF and its receptor act as a pro-atherosclerotic or anti-atherosclerotic factor. We found no association between any of the polymorphisms or haplotypes and the occurrence of cardiovascular endpoints during a 4-year follow-up period. This is in line with a meta-analysis by Chen et al, which included 5 studies that, added together, investigated the same VEGF polymorphisms as we did in relation to CAD risk and severity [16]. Also, a large mendelian randomization study by Yeung et al, which investigated 9 other VEGF SNPs, showed no evidence for a positive effect on CAD [35]. However, other studies have drawn opposite conclusions. A couple of small studies in different ethnic populations found evidence for some VEGF SNPs to be associated with CAD

[17,18,24-26]. Also, a recently published meta-analysis by Ma et al [19], which included 29 studies, found evidence for some VEGF SNPs to be associated with increased risk of CAD. The association between KDR polymorphisms and CAD has been investigated in only three case-control studies so far [13,36], that recruited Chinese patients with evidence of CAD (n=665, n=369 and n=533). Wang et al reported on two of these case-control studies and observed that all three KDR polymorphisms were associated with CAD risk [13]. Other investigators found two out of the three KDR SNPs to be associated with coronary heart disease [36].

The discrepancy with our results may firstly be due to study design, since these previous investigations consisted of case-control association studies that were limited by their small sample sizes (numbers vary from n=50 to n=800). Second, population specific differences exist with regard to underlying disease etiology and ethnicity. While previous case-control studies included a wide spectrum of CAD patients from different ethnicities, our study cohort mainly comprised of Caucasian patients. To the best of our knowledge, our analysis is the first large-scale study with long-term follow-up, that comprehensively investigated all seven functional VEGF and KDR SNPs in stable CAD patients.

Modification of ACE-inhibitor perindopril-associated treatment effect on cardiovascular outcome

The current study demonstrated that the treatment benefit of ACE-inhibitor therapy by perindopril may be modified by two variations in the VEGF gene and one variation in the KDR gene. By combining these variants into a pharmacogenetic score, we were able to create patient subgroups and predict the presence or absence of treatment benefit with perindopril for each category. We found that an increasing score with more unfavourable alleles was independently associated with a smaller benefit of perindopril treatment on cardiovascular outcome. Patients with a score >4 had no benefit from treatment with perindopril on adverse cardiovascular outcome, whereas patients with a score ≤4 experienced an enhanced 29% relative risk reduction during follow-up. Likewise, previous studies on the interaction of genetic variations with treatment response of perindopril focused on genes involved in the RAAS and KB pathway and also found some polymorphisms to be associated with treatment benefit [20, 23].

So far, the impact of genetic variation in the VEGF/KDR genes on the effect of ACE-inhibitor treatment on cardiovascular outcome has not been investigated. Therefore, we can only speculate about underlying mechanisms of the interaction between VEGF/KDR

and ACE-inhibitor therapy by perindopril. Several experimental studies have suggested that ACE-inhibitors in general might affect VEGF concentrations through some common interacting pathways. It has been demonstrated that ACE inhibition leads to enhanced activation of bradykinin signaling [37], which stimulates both the bradykinin B1 and B2 receptors on endothelial cells (Figure 1). The bradykinin B1 receptor then, in turn, stimulates VEGF expression and the bradykinin B2 receptor activates endothelial NO production [38]. Additionally, a possible interaction between the stimulated bradykinin B2 receptor and the upregulated angiotensin II type 2 receptor during ACE inhibition, may also result in enhanced VEGF expression [38]. It might therefore also be interesting to study whether angiotensin II receptor blockers, which lack these effects on bradykinin, also lack the interaction between treatment and VEGF/KDR polymorphisms. Whether ACE-inhibitors also directly influence KDR expression or functionality is not known.

Two SNPs of the VEGF (rs699947, rs2010963) and one SNP of the KDR gene (rs2305948) showed a significant incremental reduction of event rates entailed by perindopril. So far, it has not been firmly established yet as to what extent these polymorphisms influence circulating VEGF levels or KDR functionality. Relatively small and few in-vitro studies are not conclusive as to whether VEGF expression is upregulated or decelerated by certain genotypes of rs699947 and rs2010963 [14, 39-41]. Thus, based on current knowledge and evidence, we cannot fully explain the mechanisms behind the enlarged treatment benefit due to the interaction between the ACE- inhibitor perindopril and these VEGF polymorphisms. With regard to the KDR polymorphism rs2305948, located in exon 7 of the KDR gene, it is known that it encodes the extracellular region in the third NH_2-terminal Ig-like domain of the receptor, which is an important site for ligand binding [42]. A mutation at this exon results in amino acid changes, leading to a mutant KDR and thereby a reduced efficiency of VEGF binding to its receptor [13]. Although the underlying mechanism for a possible interaction between ACE-inhibitors and KDR polymorphisms has not been investigated before, we can only speculate based on our findings that ACE-inhibitors such as perindopril in some way modify KDR expression or functionality as well. Clearly, more basic research is warranted to investigate this novel concept.

Some aspects of this study warrant consideration. First, possible genetic interactions between VEGF/KDR genes and other genes, that might substantially affect cardiovascular outcome, are not addressed in our study. Second, no VEGF/KDR protein levels are available for this study, which may have strengthened our findings. Also, although the seven functional

SNPs examined in this study do not capture the entire gene and thus may not represent the complete genetic variability of the VEGF/KDR gene, we have investigated solely literature-reported functional SNPs. Furthermore, our study population consisted mainly of Caucasian male patients, meaning that interactions with gender and ethnicity could not be investigated in our study. Lastly, this study, as part of the larger EUROPA trial, focuses only on perindopril. Results may therefore not be directly extended to other ACE-inhibitors, since it is known that among ACE-inhibitors important structural and pharmacokinetic differences exist. Nevertheless, perindopril is one of the best investigated ACE-inhibitors in both preclinical and clinical studies [43].

In conclusion, this is the first and largest clinical study that has comprehensively investigated the associations between all reported functional VEGF/KDR polymorphisms and their haplotypes with 4-year cardiovascular outcome in CAD patients. Although no associations were present between any of the individual VEGF/KDR polymorphisms or their haplotypes with cardiovascular events in multivariable analysis, statistical interactions between perindopril therapy and multiple genotypes were present. A proposed pharmacogenetic score enabled us to distinguish patients with an enhanced treatment benefit from patients with no treatment benefit, independently of baseline clinical characteristics and blood pressure response. It might be speculated that by targeting therapy to those patients who would most likely benefit, the efficacy of ACE-inhibitors in stable CAD may be increased. Large future studies should further investigate this interaction.

References

1. Jespersen L. et al. Stable angina pectoris with no obstructive coronary artery disease is associated with increased risks of major adverse cardiovascular events. Eur Heart J 2012; 33: 734-744.
2. Morrow D.A. Cardiovascular risk prediction in patients with stable and unstable coronary heart disease. Circulation 2010; 121: 2681-2691.
3. Khera A.V. and Kathiresan S. Genetics of coronary artery disease: discovery, biology and clinical translation. Nat Rev Genet 2017; 18: 331-344.
4. McPherson R. and Tybjaerg-Hansen A. Genetics of Coronary Artery Disease. Circ Res 2016; 118:564-578.
5. Musunuru K. and Kathiresan S. Surprises From Genetic Analyses of Lipid Risk Factors for Atherosclerosis. Circ Res 2016; 118: 579-585.
6. Nikpay M. et al. A comprehensive 1,000 Genomes-based genome-wide association meta-analysis of coronary artery disease. Nat Genet 2015; 47: 1121-1130.
7. Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev 2004;25: 581-611.
8. Celletti F.L. et al. Vascular endothelial growth factor enhances atherosclerotic plaque progression. Nat Med 2001; 7: 425-429.
9. Hendel R.C. et al. Effect of intracoronary recombinant human vascular endothelial growth factor on myocardial perfusion: evidence for a dose-dependent effect. Circulation 2000; 101: 118-121.
10. Al-Habboubi H.H. et al. Contribution of VEGF polymorphisms to variation in VEGF serum levels in a healthy population. Eur Cytokine Netw 2011; 22: 154-158.
11. Renner W. et al. A common 936 C/T mutation in the gene for vascular endothelial growth factor is associated with vascular endothelial growth factor plasma levels. J Vasc Res 2000; 37: 443-448.
12. Ruggiero D. et al. Genetics of VEGF serum variation in human isolated populations of cilento: importance of VEGF polymorphisms. PLoS One 2011; 6: e16982.
13. Wang Y. et al. Polymorphisms of KDR gene are associated with coronary heart disease. J Am Coll Cardiol 2007; 50: 760-767.
14. Watson C.J. et al. Identification of polymorphisms within the vascular endothelial growth factor (VEGF) gene: correlation with variation in VEGF protein production. Cytokine 2000; 12: 1232-1235.
15. Petrovic D. The role of vascular endothelial growth factor gene as the genetic marker of atherothrombotic disorders and in the gene therapy of coronary artery disease. Cardiovasc Hematol Agents Med Chem 2010; 8: 47-54.
16. Chen H.L. and Liu K. Vascular endothelial growth factor polymorphisms and coronary artery disease: a systemic review and meta-analysis. Int J Cardiol 2014; 172: e220-224.
17. Han X. et al. Association between VEGF polymorphisms (936c/t, -460t/c and -634g/c) with haplotypes and coronary heart disease susceptibility. Int J Clin Exp Pathol 2015; 8: 922-927.
18. Howell W.M. et al. VEGF polymorphisms and severity of atherosclerosis. J Med Genet 2005; 42: 485 490.
19. Ma W.Q. et al. Association of genetic polymorphisms in vascular endothelial growth factor with susceptibility to coronary artery disease: a meta-analysis. BMC Med Genet 2018; 19: 108.
20. Brugts J.J. et al. Genetic determinants of treatment benefit of the angiotensin-converting enzyme inhibitor perindopril in patients with stable coronary artery disease. Eur Heart J 2010; 31: 1854-1864.
21. Brugts J.J. et al. The rationale and design of the PERindopril GENEtic association study (PERGENE): a pharmacogenetic analysis of angiotensin-converting enzyme inhibitor therapy in patients with stable coronary

artery disease. Cardiovasc Drugs Ther 2009; 23: 171-181.
22. Fox K.M. and E.U.t.O.r.o.c.e.w.P.i.s.c.A.d. Investigators, Efficacy of perindopril in reduction of cardiovascular events among patients with stable coronary artery disease: randomised, double-blind, placebo-controlled, multicentre trial (the EUROPA study). Lancet 2003; 362: 782-788.
23. Oemrawsingh R.M. et al. Individualized Angiotensin-Converting Enzyme (ACE)-Inhibitor Therapy in Stable Coronary Artery Disease Based on Clinical and Pharmacogenetic Determinants: The PERindopril GENEtic (PERGENE) Risk Model. J Am Heart Assoc 2016; 5: e002688.
24. Biselli P.M. et al. Vascular endothelial growth factor genetic variability and coronary artery disease in Brazilian population. Heart Vessels 2008; 23: 371-375.
25. Lin T.H. et al. Vascular endothelial growth factor polymorphisms and extent of coronary atherosclerosis in Chinese population with advanced coronary artery disease. Am J Hypertens 2010; 23: 960-966.
26. Cui Q.T. et al. Further evidence for the contribution of the vascular endothelial growth factor gene in coronary artery disease susceptibility. Gene 2013; 521: 217-221.
27. Kariz S. and Petrovic D. Minor association of kinase insert domain-containing receptor gene polymorphism (rs2071559) with myocardial infarction in Caucasians with type 2 diabetes mellitus: case control cross-sectional study. Clin Biochem 2014; 47: 192-196.
28. Barrett J.C. et al. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 2005; 21: 263-265.
29. Liu N. et al. Haplotype-association analysis. Adv Genet 2008; 60: 335-405.
30. Konopka A. et al. Concentration of vascular endothelial growth factor in patients with acute coronary syndrome. Cytokine 2013; 61: 664-669.
31. Liou J.Y. et al. Pericardial fluid and serum levels of vascular endothelial growth factor and endostatin in patients with or without coronary artery disease. J Formos Med Assoc 2006; 105: 377-383.
32. Inoue M. et al. Vascular endothelial growth factor (VEGF) expression in human coronary atherosclerotic lesions: possible pathophysiological significance of VEGF in progression of atherosclerosis. Circulation 1998; 98: 2108-2116.
33. Pantsulaia I. et al. Heritability of circulating growth factors involved in the angiogenesis in healthy human population. Cytokine 2004; 27: 152-158.
34. Pages G. and Pouyssegur J. Transcriptional regulation of the Vascular Endothelial Growth Factor gene - a concert of activating factors. Cardiovasc Res 2005; 65: 564-573.
35. Au Yeung S.L. et al. Vascular Endothelial Growth Factor and Ischemic Heart Disease Risk: A Mendelian Randomization Study. J Am Heart Assoc 2017; 6.
36. Li L. et al. Association of Genetic Polymorphisms on Vascular Endothelial Growth Factor and its Receptor Genes with Susceptibility to Coronary Heart Disease. Med Sci Monit 2016; 22: 31-40.
37. Ebrahimian T.G. et al. Dual effect of angiotensin-converting enzyme inhibition on angiogenesis in type 1 diabetic mice. Arterioscler Thromb Vasc Biol 2005; 25: 65-70.
38. Li P. et al. Role of bradykinin, nitric oxide, and angiotensin II type 2 receptor in imidapril-induced angiogenesis. Hypertension 2008; 51: 252-258.
39. Shahbazi M. et al. Vascular endothelial growth factor gene polymorphisms are associated with acute renal allograft rejection. J Am Soc Nephrol 2002; 13: 260-264.
40. Koukourakis M.I. et al. VEGF gene sequence variation defines VEGF gene expression status and angiogenic activity in non-small cell lung cancer. Lung Cancer 2004; 46: 293-298.
41. Steffensen K.D. et al. The relationship of VEGF polymorphisms with serum VEGF levels and progression-free survival in patients with epithelial ovarian cancer. Gynecol Oncol 2010; 117: 109-116.

42. Leppanen V.M. et al. Structural determinants of growth factor binding and specificity by VEGF receptor 2. Proc Natl Acad Sci U S A 2010; 107: 2425-2430.
43. Fox K. Contribution of perindopril to cardiology: 20 years of success. European Heart Journal Supplements 2007; 9: E10-E19.

Supplementary files

Supplementary Table 1. Occurrence of primary and secondary endpoints in the total study population, perindopril and placebo group.

Outcome endpoints	Events, n (%)		
	Total study population (n = 8711)	Perindopril group (n = 4329)	Placebo group (n = 4382)
Cardiovascular mortality, non-fatal myocardial infarction and cardiac arrest (primary endpoint)	793 (9.1)	346 (8.0)	447 (10.2)
Cardiovascular mortality	345 (4.0)	160 (3.7)	185 (4.2)
Non-fatal myocardial infarction	483 (5.5)	198 (4.6)	285 (6.5)
Non-fatal myocardial infarction or fatal myocardial infarction	534 (6.1)	219 (5.1)	315 (7.2)
Cardiovascular mortality or non-fatal myocardial infarction	784 (9.0)	342 (7.9)	442 (10.1)
Cardiovascular mortality, non-fatal myocardial infarction or revascularization (CABG or PCI)	1362 (15.6)	617 (14.3)	745 (17.0)

Supplementary Table 2. VEGF and KDR polymorphisms in relation to the secondary endpoints (n=8711).

	SNP		HR (95% CI)				
	RS number	Genotype	Cardiovascular mortality	Non-fatal myocardial infarction	Non-fatal or fatal myocardial infarction	Cardiovascular mortality or non-fatal myocardial infarction	Cardiovascular mortality, non-fatal myocardial infarction or revascularization
VEGF	rs699947	CC	Ref	Ref	Ref	Ref	Ref
		Ca	0.87 (0.67 - 1.12)	0.95 (0.76 - 1.18)	0.91 (0.74 - 1.12)	0.90 (0.76 - 1.07)	0.92 (0.80 - 1.04)
		aa	0.93 (0.69 - 1.25)	1.03 (0.80 - 1.33)	1.04 (0.82 - 1.32)	0.98 (0.80 - 1.19)	1.04 (0.90 - 1.21)
	rs1570360	GG	Ref	Ref	Ref	Ref	Ref
		Ga	0.92 (0.74 - 1.16)	0.94 (0.78 - 1.14)	0.96 (0.80 - 1.15)	0.94 (0.81 - 1.09)	0.98 (0.88 - 1.10)
		aa	0.82 (0.57 - 1.19)	0.92 (0.68 - 1.24)	0.92 (0.69 - 1.22)	0.86 (0.67 - 1.09)	0.92 (0.77 - 1.11)
	rs2010963	GG	Ref	Ref	Ref	Ref	Ref
		Gc	0.96 (0.77 - 1.20)	1.00 (0.82 - 1.20)	0.93 (0.78 - 1.11)	0.98 (0.85 - 1.14)	1.00 (0.90 - 1.12)
		cc	1.04 (0.73 - 1.47)	0.98 (0.72 - 1.34)	0.96 (0.72 - 1.29)	1.01 (0.80 - 1.28)	1.07 (0.90 - 1.28)
	rs3025039	CC	Ref	Ref	Ref	Ref	Ref
		Ct	**1.32 (1.05 - 1.66)***	0.99 (0.81 - 1.22)	0.99 (0.81 - 1.21)	1.13 (0.97 - 1.32)	1.09 (0.96 - 1.23)
		tt	0.99 (0.47 - 2.10)	1.01 (0.56 - 1.85)	1.08 (0.62 - 1.88)	0.94 (0.57 - 1.55)	1.04 (0.72 - 1.48)
KDR	rs2071559	TT	Ref	Ref	Ref	Ref	Ref
		Tc	1.10 (0.85 - 1.43)	1.00 (0.80 - 1.23)	0.97 (0.79 - 1.19)	1.02 (0.86 - 1.21)	1.06 (0.94 - 1.21)
		cc	1.10 (0.81 - 1.49)	0.93 (0.72 - 1.21)	0.93 (0.73 - 1.18)	0.98 (0.81 - 1.20)	0.95 (0.82 - 1.11)
	rs2305948	GG	Ref	Ref	Ref	Ref	Ref
		Ga	0.96 (0.72 - 1.26)	1.03 (0.82 - 1.29)	0.97 (0.77 - 1.20)	0.96 (0.80 - 1.16)	0.93 (0.81 - 1.07)
		aa	1.24 (0.51 - 3.01)	0.54 (0.17 - 1.69)	0.64 (0.24 - 1.71)	0.87 (0.43 - 1.75)	0.81 (0.47 - 1.40)
	rs1870377	AA	Ref	Ref	Ref	Ref	Ref
		At	1.14 (0.72 - 1.81)	0.99 (0.69 - 1.44)	1.07 (0.74 - 1.54)	1.03 (0.76 - 1.38)	1.15 (0.91 - 1.45)
		tt	1.08 (0.69 - 1.70)	0.95 (0.66 - 1.37)	1.03 (0.72 - 1.47)	1.01 (0.76 - 1.36)	1.15 (0.92 - 1.45)

CI=Confidence Interval; HR=Hazard Ratio; KDR=Kinase insert Domain-containing Receptor; SNP=Single Nucleotide Polymorphism; VEGF=Vascular Endothelial Growth Factor. * P-value of 0.019; all other p-values were > 0.05 (results not shown). Cox proportional hazard regression analysis was performed to estimate hazard ratios (95% confidence interval) for the secondary endpoints, adjusted for age, gender, hypertension, diabetes mellitus, smoking, hypercholesterolemia, body mass index >30, family history of coronary artery disease, previous myocardial infarction, previous stroke or peripheral vascular disease and symptomatic coronary artery disease. The homozygous common allele genotype of each SNP was used as the reference category.

Supplemental Table 3A. Haplotype analysis of the VEGF gene in relation to cardiovascular outcome.

SNP			OR (95% CI)					
rs699947	rs1570360	rs2010963	Primary endpoint	Cardiovascular mortality	Non-fatal myocardial infarction	Non-fatal or fatal myocardial infarction	Cardiovascular mortality or non-fatal myocardial infarction	Cardiovascular mortality, non-fatal myocardial infarction or revascularization
a	a	G	Reference	Reference	Reference	Reference	Reference	Reference
C	G	c	1.04 (0.91 - 1.19)	1.05 (0.86 - 1.28)	1.03 (0.87 - 1.21)	1.00 (0.85 - 1.17)	1.05 (0.92 - 1.19)	1.03 (0.93 - 1.14)
C	G	G	1.08 (0.93 - 1.26)	1.11 (0.89 - 1.40)	1.06 (0.87 - 1.28)	1.09 (0.90 - 1.30)	1.10 (0.94 - 1.28)	0.97 (0.86 - 1.10)
a	G	G	1.10 (0.94 - 1.29)	1.09 (0.87 - 1.38)	1.14 (0.94 - 1.39)	1.12 (0.93 - 1.35)	1.13 (0.97 - 1.32)	1.10 (0.97 - 1.25)

CI=confidence interval; OR=odds ratio; SNP=single nucleotide polymorphism. A probability-weighted regression analysis was applied to estimate associations between haplotypes and the primary and secondary endpoints, expressed by odds ratios with 95% confidence interval with adjustments for age, gender, hypertension, diabetes mellitus, smoking, hypercholesterolemia, body mass index >30, family history of coronary artery disease, previous myocardial infarction, previous stroke or peripheral vascular disease and symptomatic coronary artery disease. The most frequent haplotype served as the reference category.

Supplemental Table 3B. Haplotype analysis of the KDR gene in relation to cardiovascular outcome.

SNP		OR (95% CI)					
rs2305948	rs1870377	Primary endpoint	Cardiovascular mortality	Non-fatal myocardial infarction	Non-fatal or fatal myocardial infarction	Cardiovascular mortality or non-fatal myocardial infarction	Cardiovascular mortality, non-fatal myocardial infarction or revascularization
G	t	Reference	Reference	Reference	Reference	Reference	Reference
G	A	0.99 (0.86 - 1.14)	1.03 (0.84 - 1.26)	1.02 (0.85 - 1.21)	1.00 (0.85 - 1.18)	1.00 (0.87 - 1.15)	1.00 (0.90 - 1.12)
a	A	0.99 (0.79 - 1.23)	0.95 (0.68 - 1.34)	1.06 (0.81 - 1.39)	0.99 (0.76 - 1.29)	0.98 (0.78 - 1.23)	0.87 (0.72 - 1.05)
a	t	0.92 (0.66 - 1.27)	1.10 (0.70 - 1.74)	0.84 (0.55 - 1.29)	0.85 (0.56 - 1.27)	0.93 (0.67 - 1.29)	1.01 (0.78 - 1.29)

CI=confidence interval; OR=odds ratio; SNP=single nucleotide polymorphism. A probability-weighted regression analysis was applied to estimate associations between haplotypes and the primary and secondary endpoints, expressed by odds ratios with 95% confidence interval with adjustments for age, gender, hypertension, diabetes mellitus, smoking, hypercholesterolemia, body mass index >30, family history of coronary artery disease, previous myocardial infarction, previous stroke or peripheral vascular disease and symptomatic coronary artery disease. The most frequent haplotype served as the reference category.

Supplemental Table 4A. Haplotype analysis of the VEGF gene for heterogeneity of treatment effect of perindopril on cardiovascular outcome.

SNP			OR (95% CI)					
rs699947 rs1570360 rs2010963			Primary endpoint	Cardiovascular mortality	Non-fatal myocardial infarction	Non-fatal or fatal myocardial infarction	Cardiovascular mortality or non-fatal myocardial infarction	Cardiovascular mortality, non-fatal myocardial infarction or revascularization
a	a	G	Reference	Reference	Reference	Reference	Reference	Reference
C	G	c	1.34 (1.03 - 1.75)*	0.94 (0.64 - 1.39)	1.43 (1.02 - 2.00)*	1.40 (1.02 - 1.93)*	1.37 (1.05 - 1.79)*	1.24 (1.01 - 1.53)*
C	G	G	1.17 (0.85 - 1.59)	1.39 (0.88 - 2.20)	1.07 (0.72 - 1.60)	1.07 (0.74 - 1.56)	1.18 (0.87 - 1.62)	1.04 (0.81 - 1.33)
a	G	G	1.00 (0.72 - 1.37)	1.22 (0.76 - 1.94)	0.96 (0.64 - 1.43)	0.96 (0.65 - 1.40)	1.01 (0.72 - 1.39)	1.13 (0.88 - 1.44)

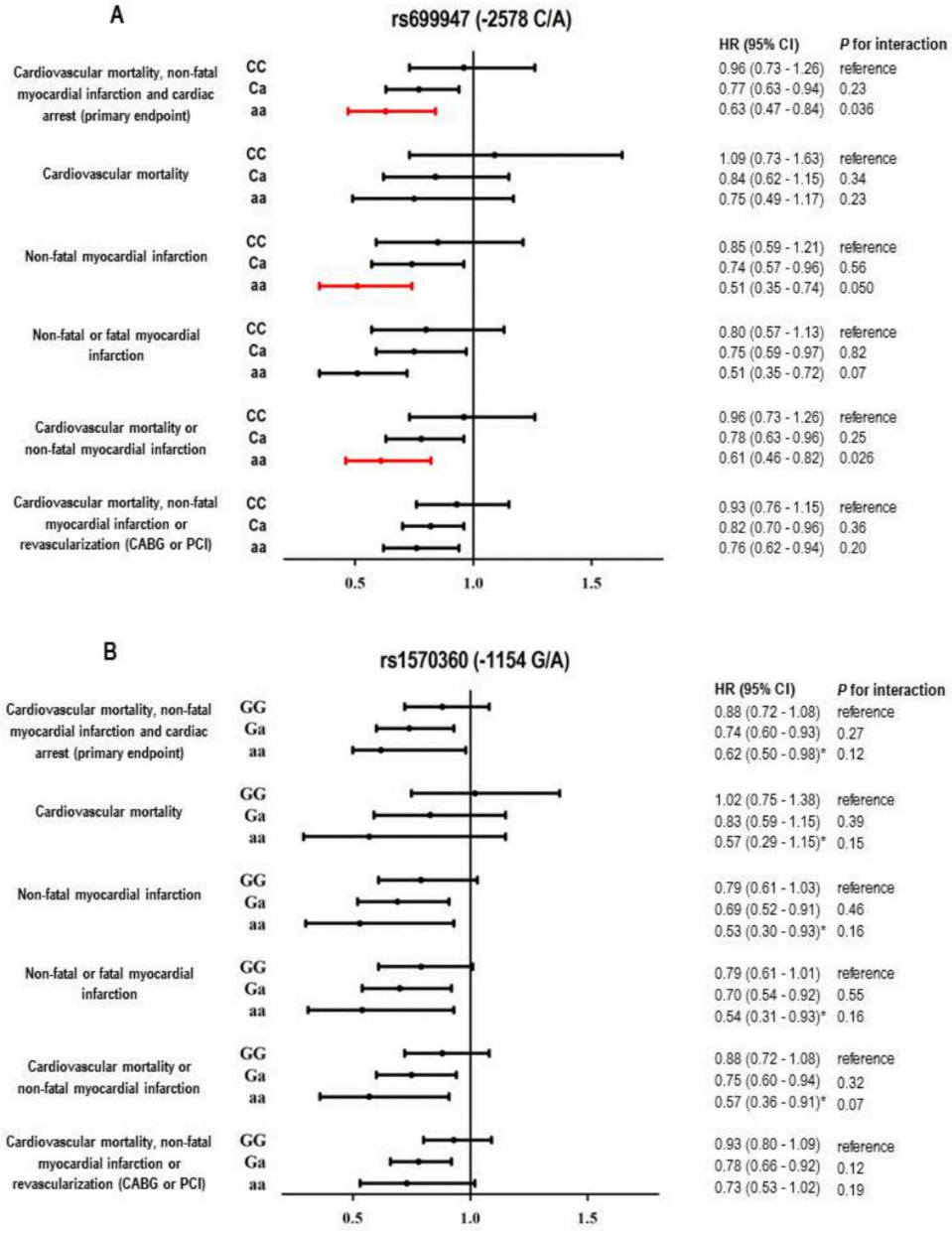
CI=confidence interval; OR=odds ratio; SNP=single nucleotide polymorphism. * P-value for interaction < 0.05; all other p-values were not significant (results not shown). A probability-weighted regression analysis was applied to estimate interaction effect sizes for the primary and secondary endpoints, expressed by odds ratios with 95% confidence interval and adjusted for age, gender, hypertension, diabetes mellitus, smoking, hypercholesterolemia, body mass index >30, family history of coronary artery disease, previous myocardial infarction, previous stroke or peripheral vascular disease and symptomatic coronary artery disease. The most frequent haplotype served as the reference category.

Supplemental Table 4B. Haplotype analysis of the KDR gene for heterogeneity of treatment effect of perindopril on cardiovascular outcome.

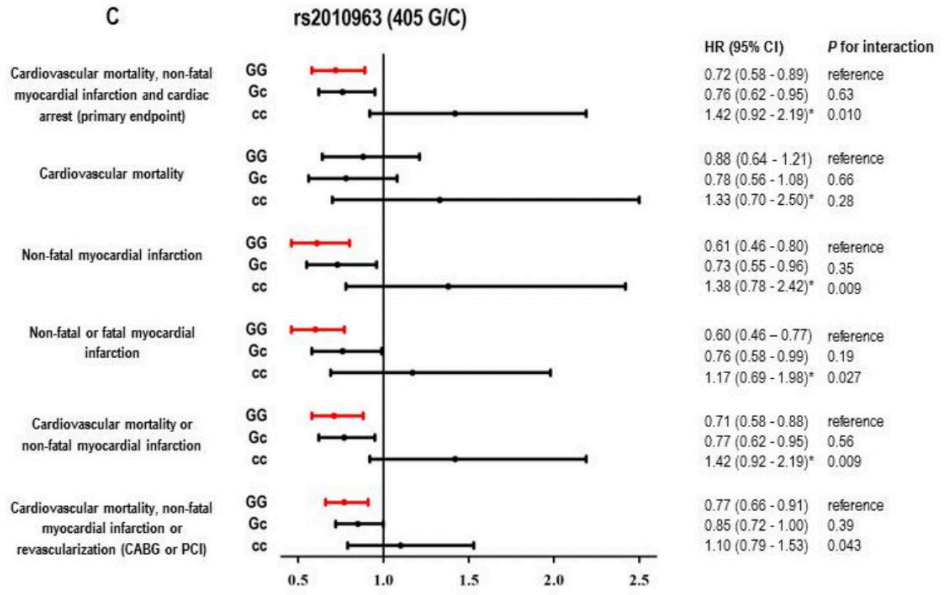
SNP		OR (95% CI)					
rs2305948 rs1870377		Primary endpoint	Cardiovascular mortality	Non-fatal myocardial infarction	Non-fatal or fatal myocardial infarction	Cardiovascular mortality or non-fatal myocardial infarction	Cardiovascular mortality, non-fatal myocardial infarction or revascularization
G	t	Reference	Reference	Reference	Reference	Reference	Reference
G	A	1.06 (0.80 - 1.40)	1.00 (0.66 - 1.51)	1.09 (0.77 - 1.54)	1.04 (0.75 - 1.45)	1.04 (0.79 - 1.38)	0.92 (0.74 - 1.15)
a	A	0.86 (0.54 - 1.35)	0.92 (0.47 - 1.83)	0.84 (0.48 - 1.46)	0.93 (0.54 - 1.59)	0.89 (0.56 - 1.40)	1.02 (0.70 - 1.48)
a	t	0.47 (0.23 - 0.94)*	0.45 (0.17 - 1.19)	0.57 (0.22 - 1.44)	0.57 (0.24 - 1.37)	0.47 (0.23 - 0.94)*	0.60 (0.36 - 0.99)*

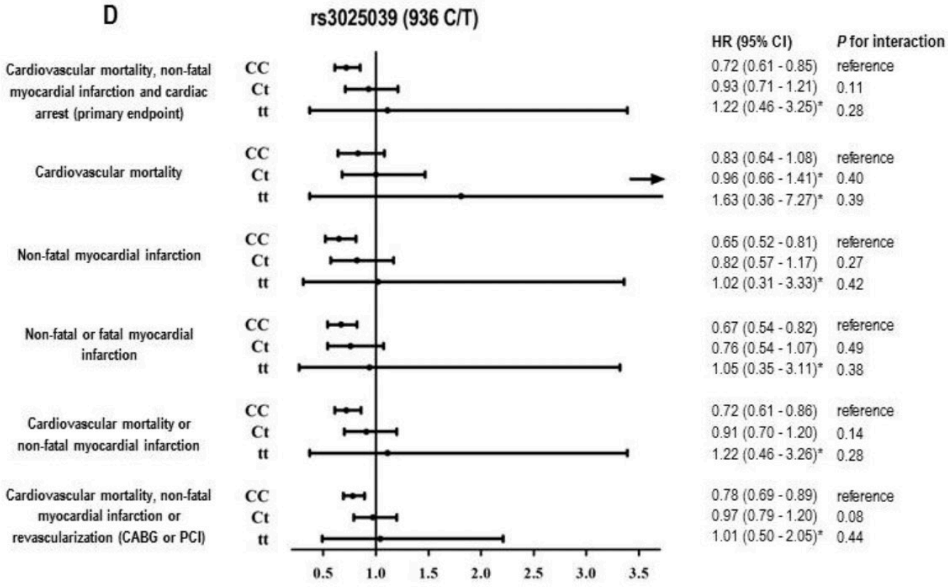
CI=confidence interval; OR=odds ratio; SNP=single nucleotide polymorphism. * P-value for interaction < 0.05; all other p-values were not significant (results not shown). A probability-weighted regression analysis was applied to estimate interaction effect sizes for the primary and secondary endpoints, expressed by odds ratios with 95% confidence interval and adjusted for age, gender, hypertension, diabetes mellitus, smoking, hypercholesterolemia, body mass index >30, family history of coronary artery disease, previous myocardial infarction, previous stroke or peripheral vascular disease and symptomatic coronary artery disease. The most frequent haplotype served as the reference category.

Supplemental Figure 1 (A - G). Modification of the treatment effect of perindopril in reducing cardiovascular outcome per genotype group of each VEGF and KDR polymorphism.

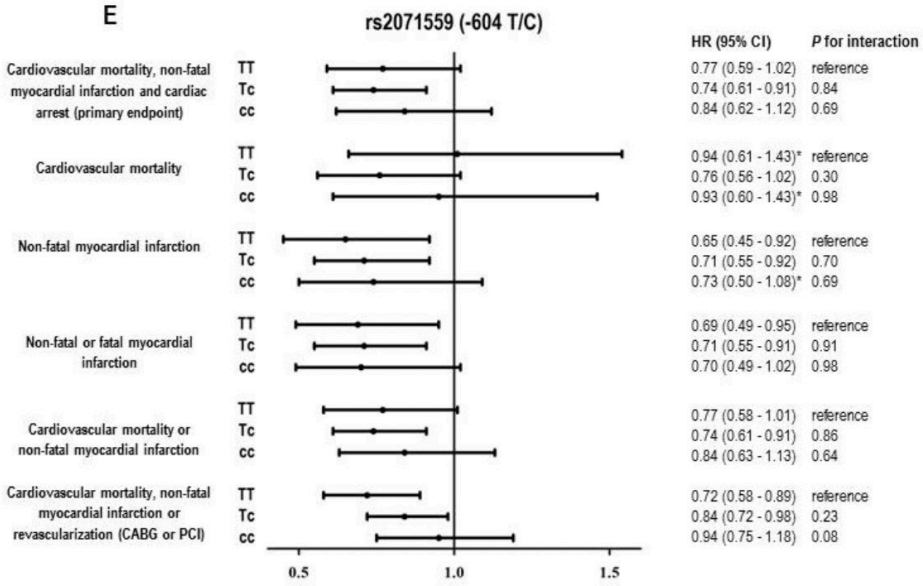


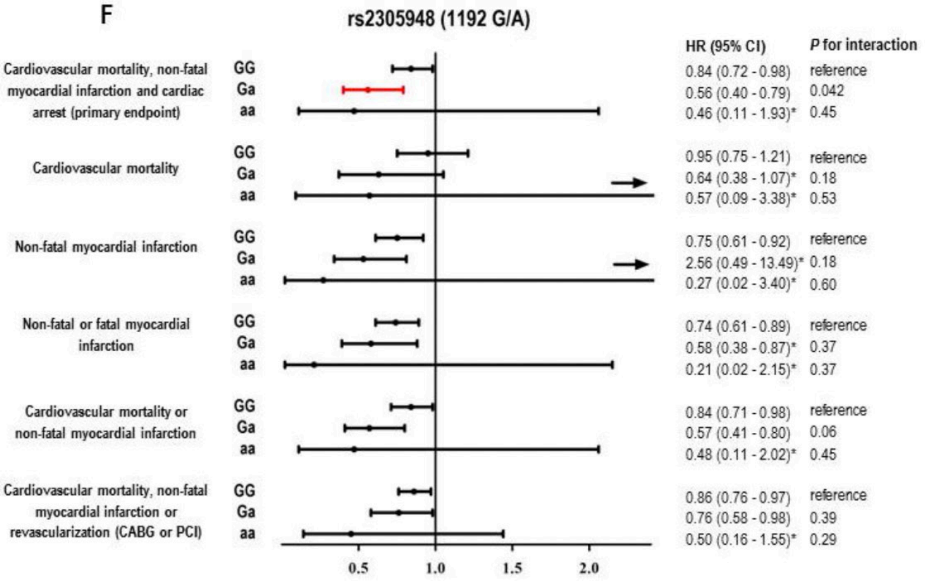
Supplemental Figure 1 (A - G) continued.



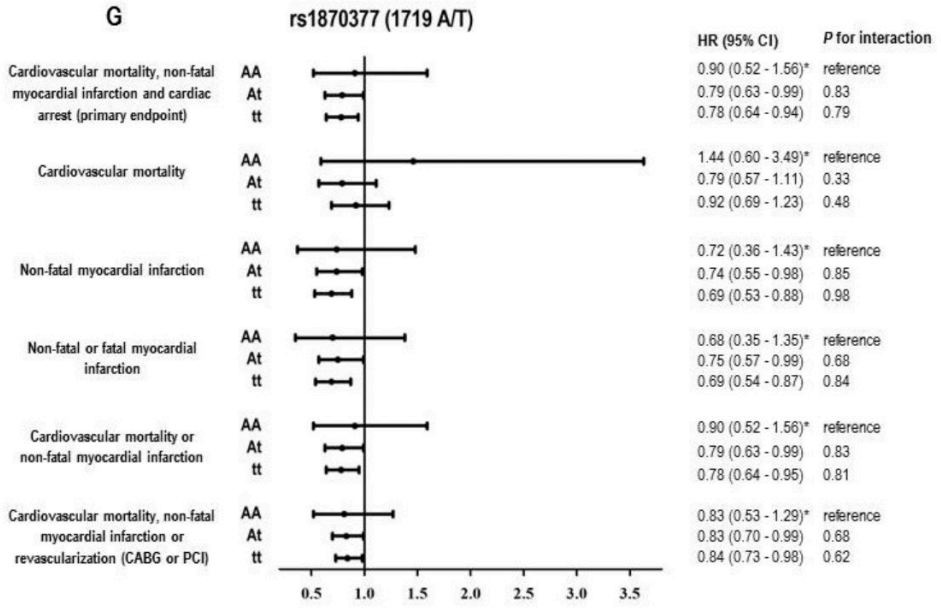


Supplemental Figure 1 (A - G) continued.

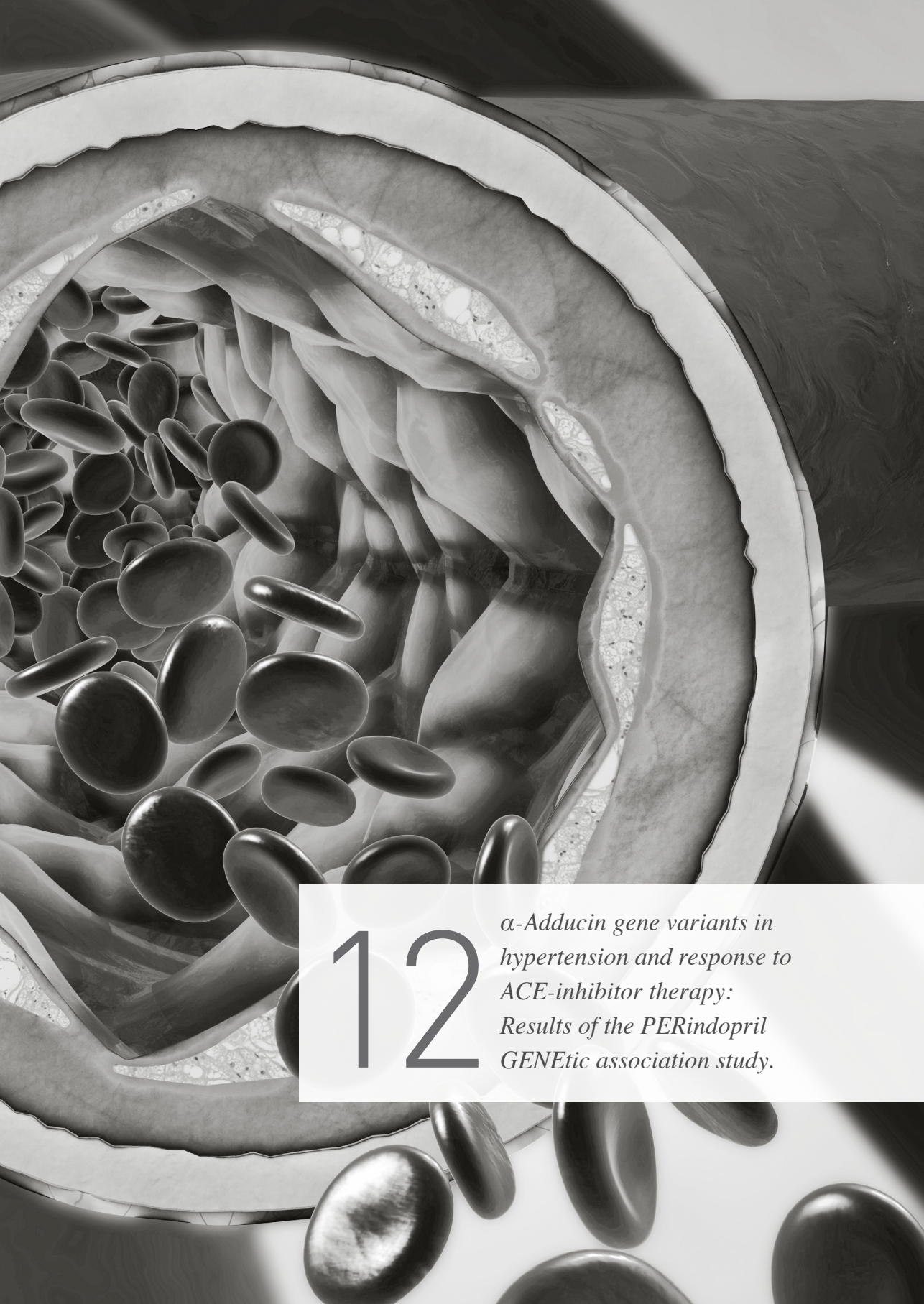




Supplemental Figure 1 (A - G) continued.



HR=Hazard Ratio. *According to current recommendations, a Cox regression model should be used with a minimum of 10 outcome events per variable. In some cases, the number of events was too low for the number of included variables in the Cox regression model. Therefore, a limited number of variables (e.g. only age and gender or only age, gender and smoking) in these cases were included as covariables in the model to estimate the hazard ratio.



12

α-Adducin gene variants in hypertension and response to ACE-inhibitor therapy: Results of the PERindopril GENEtic association study.

12

α-Adducin gene variants in hypertension and response to ACE-inhibitor therapy: Results of the PERindopril GENEtic association study.

Oemrawsingh RM, Buljubasic N, Brugts JJ, Van Vark LC, Remme WJ, Bertrand ME, Fox KM, Ferrari R, Danser AHJ, De Maat M, Simoons ML, Akkerhuis KM, Boersma E.

Submitted.

Abstract

The α-adducin (ADD1) gene may be associated with hypertension, cardiovascular outcome and treatment efficacy of diuretic therapy, but controversy exists. We evaluated the role of ADD1 in the treatment effect of ACE-inhibition. Clinical, genetic and outcome data were used from 9327 patients in the PERGENE substudy of the EUROPA-trial, a multi-center, placebo-controlled randomized trial of perindopril in patients with stable coronary artery disease during 4.2 years of follow-up. Taqman allelic discrimination was used to genotype rs4961 and rs4963, two SNPs in the ADD1 gene. The primary endpoint was a composite of cardiovascular mortality, non-fatal myocardial infarction and resuscitated cardiac arrest. Compared to the wildtype homozygotes, a 12% lower prevalence of hypertension at baseline was observed in minor allele carriers of rs4963 (adjusted OR 0.88; 95% CI 0.79-0.98 p=0.02). Blood pressure reduction was similar across all strata of rs4961 and rs4963 (p-values 0.23 and 0.61, respectively). There was no heterogeneity across ADD1 strata with respect to the incidence of the primary endpoint. The ADD1 gene polymorphisms did not modify the treatment effect of perindopril.

Introduction

According to the latest data, the estimated number of adults with hypertension increased from 594 million in 1975 to 1,1 billion in 2015 worldwide [1]. By 2025, this number is predicted to even further increase to a total of 1,6 billion [2]. Currently, hypertension already causes an estimated 7.8 million deaths per year, about 14% of the total of all annual deaths [3]. It is therefore the leading (heritable) global risk factor for mortality in the world, followed by smoking, high blood glucose, physical inactivity and obesity, which cause 5% - 9% respectively of all annual deaths [4, 5].

Apart from environmental factors, genetic variation is likely to play a crucial role in the susceptibility to hypertension [6], but may also have a role in the response or susceptibility to anti-hypertensive treatment [7]. In this line of thought, increasing interest has emerged for genetic variation in the adducin protein and its role in blood pressure (BP) regulation and hypertension. Adducin is a heterodimeric cytoskeleton protein that consists of an α-subunit, encoded by the adducin 1 (ADD1) gene, and either a β-subunit or γ-subunit according to the specific tissue, encoded by the adducin 2 (ADD2) or adducin 3 (ADD3) gene, respectively [8]. Its main function is originally described to compromise the binding of two other cytoskeleton proteins, actin and spectrin, playing an important role in maintenance of plasma cell membrane integrity [8, 9]. Further it has been discovered to function in signal transduction handling cell sodium transport in renal tubular cells [8, 10]. Functional point mutations within the gene coding for the α-adducin subunit lead to the stimulation of renal sodium transport by activation of Na-K-ATPase at the basolateral membrane, apparently resulting in enhanced tubular sodium reabsorption [8, 10, 11], hypertension [12-15] and adverse cardiovascular events [16]. Particular attention has been drawn by the Gly460Trp (rs4961) and Ser586Cys (rs4963) variants, which are both located on the ADD1 gene (chromosome 4p16.3) and lead to amino acid substitutions of the α-subunit protein. Several large scale studies, however, failed to demonstrate an association between ADD1 polymorphisms and hypertension or adverse cardiovascular outcome [17-20].

When considering adducin function in tubular pathophysiology, and its potential association with hypertension and even adverse outcomes, several attempts have been undertaken to investigate whether antihypertensive medication, in particular diuretics, can modify the cardiovascular risk across various adducin genetic strata [21-27]. The results of these studies on interaction between ADD1 polymorphisms and the treatment effect of

several diuretics are also conflicting. Other antihypertensives have not been studied at large with regard to their interaction with adducin polymorphisms on cardiovascular outcome. The GenHAT investigators studied the ACE-inhibitor lisinopril (next to amlodipine, chlortalidone and doxazosin), just as Schelleman et al studied ACE-inhibitors as a group in the observational Rotterdam Study, but these studies did not contain a placebo arm [23, 26]. Hence, no conclusions could be drawn on the genotype-ACE-inhibitor interaction and the net effect on endpoints. However, it is important to study the relation between adducin polymorphisms and the net effect of ACE-inhibitor therapy versus placebo. Adducin seems to be involved in tubular sodium reabsorption and hypertension, whereas ACE-inhibitor therapy results in quite the opposite effect, namely inhibition of aldosteron secretion and, as a consequence, natriuresis and BP lowering.

Furthermore, no studies have investigated the association between the ADD1 polymorphisms and ACE-inhibitor use in a large cohort of patients with stable coronary artery disease (CAD). According to guidelines, ACE-inhibitors are widely prescribed to this population [28]. The degrees of response, however, differ for each individual patient and genetic determinants may be used to predict treatment benefit [7].

The PERindopril GENEtic Association study (PERGENE), a substudy of the EUROPA trial, offers an unique opportunity to verify pharmacogenetic relationships in a large randomized cohort of patients with stable CAD randomized to ACE-inhibitor therapy or placebo. In the present study, we investigated whether ADD1 gene variants were related to hypertension and (an increased) cardiovascular risk in patients with stable CAD. In addition, we investigated whether the ADD1 gene variant modified the response to ACE-inhibitor therapy in terms of BP reduction or reduction in adverse cardiovascular adverse events during follow-up.

Methods

Study population and design

The designs of both the PERGENE and EUROPA study have been reported previously [29, 30]. In brief, EUROPA was a randomized, double-blinded, placebo-controlled trial designed to assess the effect of perindopril (8 mg daily) on the combined primary endpoint of cardiovascular mortality, non-fatal myocardial infarction (MI) and resuscitated cardiac

arrest in 12218 patients with stable CAD, but without overt heart failure or uncontrolled hypertension. The use of perindopril resulted in a 20% relative risk reduction (adjusted HR 0.80, 95% CI: 0.71-0.91) in the rate of the primary endpoint during a mean follow-up of 4.2 years [29]. This study was approved by the Institutional Review Boards of all participating sites and was performed in accordance to the Declaration of Helsinki. Written informed consent for genetic association analyses was obtained from all patients. A DNA bio-bank was established within the EUROPA trial for the purpose of the PERGENE substudy, which investigates whether genetic variation is a determinant of the risk of future adverse cardiovascular outcome and / or treatment benefit by the use of perindopril [7, 30].

Genotyping

DNA was successfully isolated in 9454 patients, using an automated isolation process [30]. The ADD1 single nucleotide polymorphisms (SNP), rs4961 and rs4963 were assessed with the use of Taqman allelic discrimination assays (Applied Biosystems, Carlsbad, CA, USA). Genotype call rates for rs4961 and rs4963 were 98.9% and 99.0%, resulting in 9346 and 9364 patients respectively. 9327 patients (98.6%) had genotype information for both ADD1 gene polymorphisms. Hardy-Weinberg equilibrium was tested with chi-square analysis. Repeated laboratory analyses were performed in a random group of samples (5%), resulting in a >99% duplicate concordance rate.

Statistical analysis

Analyses were based on the intention-to-treat principle. Blood pressure reduction was calculated as the mean difference in BP between screening visit 1 and randomization, which took place after a 4-week run-in period in which all patients were treated with perindopril. One-way ANOVA was used to calculate P-values for the differences in BP reduction across genotype strata. Hypertension at baseline was defined as a BP above 140/95 mmHg or the use of anti-hypertensive medication, according to the definition in the study protocol of the main study [29]. Rs4961 and the prevalence of hypertension at baseline and ACE-inhibitor treatment effect were studied with patients either classified as ADD1 wild type homozygotes (Gly460Gly) or as ADD1 variant carriers (Gly460Trp or Trp460Trp), because of the rarity of the Trp460Trp genotype (3.5%). The same applies to rs4963 in which Cys586Cys carriers only constituted 2.9% of the study population. Logistic regression analysis was used to estimate odds ratios and 95% confidence intervals

(CI) for the relation between genotype, the prevalence of hypertension at baseline and the occurrence of systolic BP reduction on perindopril during the run-in period. Three models are used throughout the manuscript: an unadjusted model, a model adjusted for age and gender, and a "full model" according to a risk prediction model based upon the EUROPA trial [31]. The full model adjusts for age, gender, systolic BP, creatinine clearance, total cholesterol levels, previous stroke or peripheral artery disease, obesity (BMI>30 kg/m^2), current smoking, symptomatic CAD, diabetes mellitus, previous MI, family history of CAD, previous revascularization and active treatment with perindopril. Cox regression analysis was used to estimate hazard ratios for the risk of the primary endpoint (cardiovascular mortality, non-fatal MI, or resuscitated cardiac arrest) during follow-up. Since the ADD1 gene appeared to be associated with hypertension, a subgroup analysis of patients with and without hypertension was performed. The treatment benefit of perindopril in terms of the relative risk reduction of the primary endpoint was evaluated for the wild type homozygotes and variant carriers of each SNP. Genotype-treatment interaction was assessed by including treatment, genotype and treatment*genotype in the full model. All statistical tests were two-sided with a type I error level of 0.05. Analyses were performed with IBM SPSS statistics version 24.0

Results

In total, 9327 patients (98.6% of the total number of 9454 patients of whom DNA was isolated in the PERGENE study) had genotype information for both ADD1 gene polymorphisms, of whom 4667 (50.0%) were randomized to perindopril and 4660 to placebo. Baseline characteristics of the study population are provided in Table 1. Median follow-up was 4.2 years (interquartile range 4.0-4.5 years). Males constituted 85.6% of the study population and 98.9% of participants were of Caucasian descent. Fifty percent of participants were randomised to perindopril and treatment with perindopril was protective against the primary endpoint (adjusted HR 0.79, 95% CI: 0.69-0.91). Hypertension at baseline was present in 28% of the study population.

Table 1. Baseline characteristics of the study population.

Number of patients[a]	9327
Age, years	59.8 (9.3)
Male gender (n, %)	7984 (85.6)
Hypertension (n, %)	2612 (28.0)
Diabetes mellitus (n, %)	1215 (13.0)
Hypercholesterolemia (n, %)[b]	5876 (63.0)
Current smoking (n, %)[c]	1399 (15.0)
Obesity (BMI>30 kg/m^2) (n, %)	1970 (21.1)
Symptomatic CAD (n, %)[d]	2350 (25.2)
Family history of CAD (n, %)	2516 (27.0)
Previous MI (n, %)	6063 (65.0)
Previous revascularisation (n, %)	5505 (59.0)
Previous stroke or PAD (n, %)	832 (8.9)
Medication	
Platelet-inhibitors (n, %)	8584 (92.0)
Beta-blockers (n, %)	5877 (63.0)
Lipid-lowering agents (n, %)	5129 (55.0)
Calcium-antagonists (n, %)	2908 (31.2)
Systolic blood pressure (mmHg)	136.9 (15.2)
Diastolic blood pressure (mmHg)	81.8 (8.1)
Creatinine clearance (μmol/L)[e]	86.3 (25.7)
Total cholesterol (mmol/L)	5.4 (1.0)
Randomization, allocation to perindopril (n,%)	4667 (50.0)
Primary endpoint (n,%)	803 (8.6)
Systolic BP reduction by perindopril (mmHg)[f]	-8.7 (14.6)
Diastolic BP reduction by perindopril (mmHg)[f]	-4.0 (8.6)

BP=blood pressure; CAD=coronary artery disease; MI=myocardial infarction; PAD=peripheral artery disease. Summary statistics for continuous variables are presented as mean (standard deviation). Categorical data are summarized as number (percentages).

[a] Number of patients with complete data of both SNPs.

[b] Previously known total cholesterol >6·5 mmol/L or receiving lipid-lowering treatment.

[c] Use of tobacco within the last month.

[d] Stable angina pectoris or history of congestive heart failure.

[e] Estimation by Cockroft-Gault equation.

[f] Blood pressure reduction was calculated as the mean difference in blood pressure during a 4-week run-in period in the EUROPA trial, which took place prior to randomization and in which all patients were treated with perindopril.

Both SNPs were in Hardy-Weinberg equilibrium. Genotype frequencies of rs4961 were GG in 66.1%, GT in 30.4% and TT in 3.5% of the patients, which results in a minor allele frequency of 18.7%. Genotype frequencies of rs4963 were CC in 68.3%, CG in 28.8% and GG in 2.9% of the patients respectively, with a minor allele frequency of 17.3% (Table 2).

Table 2. Genotype frequencies of rs4961 and rs4963 in the ADD1 gene.

SNP	Location	Allele	Amino acid change	Genotype frequencies			Minor allele freq	Hardy-Weinberg
Rs4961	Exon 10	G/T	Gly-460-Trp	66.1 %	30.4 %	3.5 %	18.7 %	ns
Rs4963	Exon 13	C/G	Ser-586-Cys	68.3 %	28.8 %	2.9 %	17.3 %	ns

ns=non-significant.

Rs4961 was not associated with the prevalence of hypertension at baseline (Table 3). Minor allele carriers tended to have a lower prevalence of hypertension, but the association was non-significant. However, the ADD1 gene variant rs4963 was significantly associated with the prevalence of hypertension in patients with stable CAD, both in univariate and multivariate analysis (Table 3). Minor allele carriers of rs4963 had a 12% lower prevalence of hypertension compared to the wild type homozygotes (adjusted OR 0.88; 95% CI 0.79-0.98).

Table 3. ADD1 gene polymorphisms and prevalent hypertension at baseline.

		Unadjusted model	Adjusted for age and gender only	Full model
		OR (95% CI)	**OR (95% CI)**	**OR (95% CI)**
Rs4961	GG (66.1%)	1.00	1.00	1.00
	GT+TT (33.9%)	0.92 (0.83-1.01)	0.91 (0.83-1.01)	0.91 (0.82-1.01)
Rs4963	CC (68.3%)	1.00	1.00	1.00
	CG+GG (31.7%)	0.89 (0.81-0.99)	0.89 (0.81-0.98)	0.88 (0.79-0.98)

Mean systolic and diastolic BP reductions during a 4 week run-in period prior to randomization, in which all patients were treated with perindopril, were 8.7 and 4.0 mmHg, respectively (Table 1). Blood pressure reduction by the use of perindopril was not modified across genotype strata of rs4961 and rs4963 as presented in Table 4, also not within subgroups of hypertension status (data not shown).

During the run-in phase of the EUROPA trial, systolic BP reduction on perindopril was observed in 77.4% of all patients. ADD1 polymorphisms did not predict whether systolic BP reduction occurred or not (Table 5). The full model revealed that younger age, female sex, obesity, higher systolic BP at baseline, lower total cholesterol, diabetes mellitus and a history without a previous MI were predictive for systolic BP reduction on perindopril. In total, 803 patients (8.6% of the study population) experienced the primary endpoint of cardiovascular mortality, non-fatal MI or resuscitated cardiac arrest during follow-up.

Analysis of different genotype strata across rs4961 and rs4963 did not reveal any association between ADD1 genotype and the primary endpoint (Table 6). ADD1 wild type homozygotes and ADD1 variant carriers had an equal risk on the occurrence of the primary endpoint, both within the total study population, as within subgroups based on hypertension status (Table 6).

Table 4. ADD1 gene polymorphisms and blood pressure reduction on perindopril during the 4 week run-in phase of the EUROPA trial.

		Systolic BP reduction (mmHg)	P-value	Diastolic BP reduction (mmHg)	P-value
Rs4961	GG (66.1%)	- 8.5 (14.5)	0.11	- 4.0 (8.6)	0.23
	GT (30.4%)	- 8.9 (14.7)		- 4.1 (8.5)	
	TT (3.5%)	- 9.7 (15.5)		- 4.0 (8.5)	
Rs4963	CC (68.3%)	- 8.5 (14.5)	0.75	- 4.0 (8.6)	0.61
	GC (28.8%)	- 9.0 (14.7)		- 4.2 (8.5)	
	GG (2.9%)	- 9.6 (15.1)		- 3.8 (8.5)	

Blood pressure reduction was calculated from the difference in blood pressure measured at the baseline screening visit and the blood pressure measured at randomization after the 4 week run-in period, in which all patients were treated with the ACE-inhibitor perindopril. BP indicates blood pressure.

Table 5. ADD1 gene polymorphisms and occurrence of SBP reduction on perindopril during the 4 week run-in phase of the EUROPA trial.

		Unadjusted model	Adjusted for age and gender only	Full model
		OR (95% CI)	**OR (95% CI)**	**OR (95% CI)**
Rs4961	GG (66.1%)	1.00	1.00	1.00
	GT+TT (33.9%)	1.05 (0.95-1.17)	1.05 (0.95-1.17)	1.03 (0.92-1.16)
Rs4963	CC (68.3%)	1.00	1.00	1.00
	CG+GG (31.7%)	1.07 (0.96-1.19)	1.07 (0.96-1.19)	1.05 (0.93-1.17)

Table 6. ADD1 gene polymorphisms and incident risk of the primary endpoint (cardiovascular mortality, myocardial infarction, resuscitated cardiac arrest) during 4 years of follow-up.

		Total study Population	Patients without hypertension (72%)	Patients with hypertension (28%)
		HR (95% CI)	**HR (95% CI)**	**HR (95% CI)**
Rs4961	GG (66.1%)	1.00	1.00	1.00
	GT+TT (33.9%)	0.99 (0.86-1.16)	1.00 (0.83-1.20)	1.02 (0.80-1.31)
Rs4963	CC (68.3%)	1.00	1.00	1.00
	CG+GG (31.7%)	0.99 (0.85-1.15)	1.00 (0.83-1.20)	1.00 (0.78-1.29)

Multivariate Cox regression analysis was used to estimate hazard ratios for the risk of the primary endpoint. Adjustments took place according to the "full model" which includes age, gender, systolic blood pressure, creatinine clearance, total cholesterol levels, previous stroke or peripheral artery disease, obesity (BMI>30 kg/m^2), current smoking, symptomatic CAD, diabetes mellitus, previous MI, family history of CAD and previous revascularization.

With regard to the treatment effect of perindopril, risk reductions across different ADD1 strata were in concordance with the overall perindopril treatment effect and ranged between 19 and 26% (adjusted HRs ranged from 0.74 (0.57-0.97) to 0.81 (0.69-0.96), Figure 1). Thus, treatment benefit of perindopril on the occurrence of the primary endpoint was not additively modified by different strata of rs4961 and rs4963 (Figure 1). This is also demonstrated by the fact that no interaction between ADD1 genotype and treatment effect was found (P for interaction for rs4961 and rs4963 were 0.63 and 0.74, respectively).

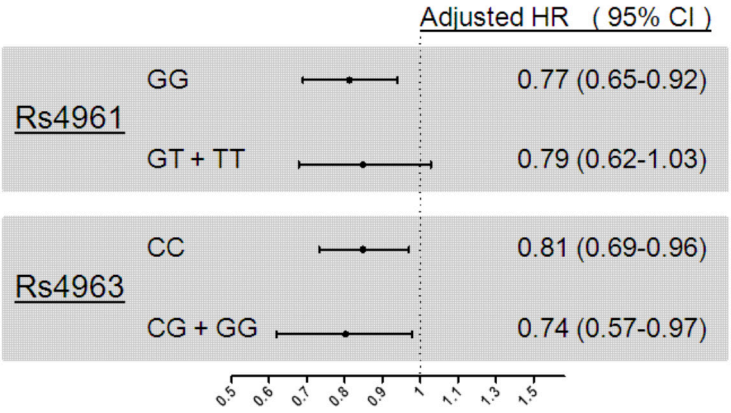


Figure 1. ADD1 gene polymorphisms and treatment benefit of perindopril.

Discussion

Our study is the first to investigate the relation between α-adducin (ADD1) gene polymorphisms, hypertension and ACE-inhibitor treatment benefit (versus placebo) on BP reduction and cardiovascular outcome in a large study population with stable CAD. The main findings are that the ADD1 rs4961 (Gly460Trp) polymorphism was not associated with the prevalence of hypertension at baseline. In contrast, minor allele carriers of the ADD1 rs4963 (Ser586Cys) polymorphism had a significantly lower prevalence of hypertension than the wildtype homozygotes. Furthermore, ADD1 polymorphisms were not associated with cardiovascular outcome in terms of cardiovascular mortality, non-fatal MI or resuscitated cardiac arrest during 4.2 years of follow-up. Nor did they modify the treatment effect of perindopril in terms of BP reduction or cardiovascular risk

reduction in a large population of stable CAD patients. Our data add to a growing body of evidence on adducin and its role in hypertension, cardiovascular risk and interaction with antihypertensive treatment.

Adducin polymorphisms and prevalent hypertension

The data that have been published on ADD1 polymorphisms and the prevalence of hypertension so far have been conflicting, not only in terms of whether such an association actually exists, but also on whether the minor or the major allele imposes a larger risk on hypertension. In 1995, presumptions of the link between ADD1 polymorphisms and hypertension based on experimental data on rats, have been reported in humans for the first time [32]. Shortly after that, this hypothesis was confirmed by Cusi et al, who conducted an European linkage study with 137 hypertensive sibling-pairs and showed that the α-adducin locus was linked to hypertension in human beings [13]. Specifically, they concluded that the rs4961 minor allele was associated with a higher prevalence of essential hypertension and a greater sensitivity to changes in sodium balance among patients with a salt-sensitive form of essential hypertension [12]. Subsequent studies investigating the rs4961 polymorphism in relation to hypertension found positive associations with minor allele carriers as well [12, 33]. Another association between α-adducin polymorphism rs4961 and BP was found in the GenHAT study (an ALLHAT substudy), in which all patients were hypertensive, ≥ 55 years old, and all had at least one additional risk factor for CAD [23]. Within the subgroup of 3598 patients who were not taking antihypertensive medication at study entry, there was a difference in diastolic BP of 2.2mmHg between certain adducin polymorphisms. Remarkably, in this study, the highest diastolic BP was not found in the minor allele carriers, but in the wildtype (Gly460Gly) homozygotes [23]. This finding somewhat resembles our result that the rs4963 minor allele carriers had a significantly lower prevalence of hypertension than the wildtype (Ser586Ser) homozygotes. The r^2 measure of linkage disequilibrium between rs4961 and rs4963 in the International 1000 Genomes Project is 0.75 (in Caucasians with ancestry from Northern and Western Europe; CEU). Strikingly, to the best of our knowledge, there are no studies reporting on a relationship between the rs4963 SNP and BP in Caucasians. One meta-analysis (n=12 studies) has reported on the positive association between rs4963 polymorphism and hypertension, but solely in Asian populations [14]. Hence, our study is the *first* to demonstrate an association between rs4963 and prevalence of hypertension in a Caucasian population.

Apart from the studies mentioned above, there are many other studies that failed to demonstrate any relation between adducin gene polymorphisms and hypertension [17, 19, 22]. There are several explanations for the inconsistencies that have been found so far. First of all, from a pathophysiologic point of view: hypertension is not only a complex, but also a multi-phase and multifactorial disease. The proposed mechanism of action with regard to adducin genotypes in hypertension depends on activation of the Na-K-ATPase pump at the basolateral membrane of tubular cells, thus resulting in sodium retention and expansion of circulating volume [21]. It is obvious, however, that renal sodium preservation alone is not the only determinant in the development of hypertension. Dietary sodium intake, compensatory hormonal and renal mechanisms and the genetic and non-genetic determinants of vascular tone for instance, are at least equally important [34]. Secondly, genetic heterogeneity (the same phenotype may be produced by different genetic mechanisms) and epistasis (the effect of a particular gene is modulated by other genes) may be important confounders in the relation between the adducin gene and hypertension [34, 35]. Thirdly, it is important to put each and every study on the association between the adducin gene and hypertension into an epidemiologic perspective. Different study populations with vastly different characteristics (e.g. exclusively hypertensive populations versus populations with a minority of hypertensives etc.) and varying sample sizes have yielded different results. In this regard it is also interesting to highlight the importance of ethnicity. Data from the International 1000 Genomes Project clearly indicates that genotype and allele frequencies of rs4961 and rs4963 differ across various populations. For example, the alleles that are considered the minor alleles of rs4961 and rs4963 in this study, as well as in Caucasians with ancestry from Northern and Western Europe (CEU) within 1000 Genomes, appear, in contrast, in the *majority* of Han Chinese (CHB) and Japanese (JPT) subjects [36]. Such a different genetic distribution may explain why associations with hypertension and cardiovascular outcome may be apparent in certain populations and not in others. This is endorsed by two recently published meta-analyses, showing that rs4961 minor allele carriers had higher risk of hypertension only among Asian subjects, but not Caucasians [15, 37].

Adducin polymorphisms, cardiovascular outcome and interaction with antihypertensive treatment

The INVEST-GENES investigators have published that in exclusively hypertensive patients with CAD (n=5979), a subgroup of Afro-American carriers of the minor Trp460 variant

had an impressive 8-fold increased risk of death, compared to carriers of the major Gly460 variant [16]. On the other hand, no association with hypertension and adverse outcome was found within a large Dutch case-control study [26]. These latter findings are in line with our analysis which also demonstrated a lack of association between ADD1 polymorphisms and blood pressure response or cardiovascular outcome.

In an analysis from the "Group Health Cooperative", diuretic therapy was associated with a 51% reduction in the occurrence of non-fatal MI or stroke in 385 carriers of the ADD1 minor variant, whereas no relation between outcome and diuretic use was found in the 653 major variant carriers [22]. Analyses from the randomized controlled trials INVEST-GENES and GenHAT (ALLHAT substudy), however, showed no interaction between ADD1 and diuretic therapy on cardiovascular outcome in much larger sample sizes of respectively 5979 and 13269 participants [16, 23]. Furthermore, a genome-wide association meta-analysis (1.1 million SNPs) did not identify any interaction between ADD1 and blood pressure response on thiazide diuretics [38]. Several observational cohort studies have shown similar results [24-27].

The interaction between ADD1 and ACE-inhibition on cardiovascular outcome has not been studied in a placebo-controlled setting before [23, 26]. This has limited any conclusion with respect to the net effect of ACE-inhibition on clinical cardiovascular outcome across various ADD1 genotype strata. Ideally, genetic heterogeneity in the treatment benefit of ACE-inhibitor therapy could lead to a more individually-tailored treatment regimen. In the EUROPA trial, perindopril reduced the *relative* risk of cardiovascular mortality, MI, and resuscitated cardiac arrest by 20% [29]. However, *absolute* risk reduction, which directly influences the numbers needed to treat, was 1.9%. Several prior approaches to tailor ACE-inhibitor therapy based upon clinical characteristics have been unsuccessful. Identification of those patients most likely to benefit from ACE-inhibition versus those that are less likely to benefit, has been difficult.

Our analyses have demonstrated that BP reduction was not modified by the various ADD1 genotype strata during the run-in phase of the EUROPA trial (in which all participants were treated with perindopril during four weeks). This finding alone should not immediately have to lead to the conclusion that there is no interaction between ADD1 genotype strata and treatment benefit of perindopril, since a post-hoc analysis of the EUROPA trial demonstrated that the treatment benefit of perindopril could not be fully explained by baseline BP or BP reduction with perindopril [39]. Unfortunately, our

further analyses on ADD1 genotype revealed that the treatment effect of perindopril on the primary endpoint was not modified cross genotype strata of rs4961 and rs4963 during the placebo controlled follow-up of 4.2 years. As treatment benefit of ACE-inhibition was similar across the various genotype strata, our data do not provide a window of opportunity for possible future individualized tailoring of ACE-inhibitor therapy in patients with stable CAD.

Our study has particular strengths to note. The sample size is large, follow-up is relatively long and various additional qualities of a well-designed placebo-controlled double-blinded RCT, such as high quality phenotypical data and independent event adjudication are apparent. Furthermore, it is the only study so far to investigate the treatment effect of ACE-inhibition across various ADD1 genotype strata in a placebo-controlled setting, thus enabling conclusions on the treatment effect of the ACE-inhibitor alone. It contains both hypertensive and non-hypertensive individuals, thus enabling investigation of the prevalence of hypertension and ADD1 genotypes. Ultimately, it is the first to present data on rs4963 and prevalence of hypertension, cardiovascular outcome and ACE-inhibitor treatment benefit in Caucasians.

Our study should be interpreted within the context of its potential limitations. The analysis of two SNPs is obviously not the only or most conclusive way to determine genetic variability on ADD1. Further research on the exact role of ADD1 in hypertension and cardiovascular outcome should be warranted, ideally in combination with certain recently-found effect modifiers such as γ-adducin (ADD3) [35]. Finally, the generalizability of our results is limited, since the EUROPA trial mainly consisted of Caucasian men. As discussed above, different genetic distribution may explain why associations with hypertension and cardiovascular outcome may be apparent in certain, e.g. Afro-American and Chinese populations, but not within our study population.

In conclusion, based on our study on the ADD1 polymorphisms rs4961 and rs4963 in 9327 patients with stable CAD, only minor allele carriers of the rs4963 (Ser586Cys) polymorphism have a significantly lower prevalence of hypertension than the wildtype homozygotes. Furthermore, ADD1 polymorphisms are not associated with cardiovascular outcome, neither do they modify the treatment effect of perindopril in terms of BP reduction or cardiovascular risk reduction in a Caucasian population of stable CAD patients.

References

1. Worldwide trends in blood pressure from 1975 to 2015: a pooled analysis of 1479 population-based measurement studies with 19.1 million participants. Lancet 2017; 389: 37-55.
2. Kearney P.M. et al. Global burden of hypertension: analysis of worldwide data. Lancet 2005; 365: 217-223.
3. Forouzanfar M.H. et al. Global Burden of Hypertension and Systolic Blood Pressure of at Least 110 to 115 mm Hg, 1990-2015. JAMA 2017; 317: 165-182.
4. Global, regional, and national comparative risk assessment of 84 behavioural, environmental and occupational, and metabolic risks or clusters of risks for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet 2018; 392: 1923-1994.
5. World Health Organization. Global Health Risks: Mortality and Burden of Disease Attributable to Selected Major Risks.
6. Warren H.R. et al. Genome-wide association analysis identifies novel blood pressure loci and offers biological insights into cardiovascular risk. Nat Genet 2017; 49: 403-415.
7. Brugts J.J. et al. Genetic determinants of treatment benefit of the angiotensin-converting enzyme-inhibitor perindopril in patients with stable coronary artery disease. Eur Heart J 2010; 31: 1854-1864.
8. Bianchi G.P. Ferrari and Staessen J.A. Adducin polymorphism: detection and impact on hypertension and related disorders. Hypertension 2005; 45: 331-340.
9. Mische S.M. et al. Erythrocyte adducin: a calmodulin-regulated actin-bundling protein that stimulates spectrin-actin binding. J Cell Biol 1987; 105: 2837-2845.
10. Staessen J.A. and Bianchi G. Adducin and hypertension. Pharmacogenomics 2005; 6: 665-669.
11. Manunta P. et al. Adducin polymorphism affects renal proximal tubule reabsorption in hypertension. Hypertension 1999; 33: 694-697.
12. Li Y.Y. Alpha-Adducin Gly460Trp gene mutation and essential hypertension in a Chinese population: a meta-analysis including 10,960 subjects. PLoS One 2012; 7: e30214.
13. Cusi D. et al. Polymorphisms of alpha-adducin and salt sensitivity in patients with essential hypertension. Lancet 1997; 349: 1353-1357.
14. Qu Y.L. et al. Association between alpha-adducin gene rs4963 polymorphism and hypertension risk in Asian population: a meta-analysis. Cell Mol Biol 2016; 62: 62-64.
15. Jin H.Y. Huang and Yang G. Association between alpha-adducin rs4961 polymorphism and hypertension: A meta-analysis based on 40 432 subjects. J Cell Biochem 2019; 120: 4613-4619.
16. Gerhard T. et al. Alpha-adducin polymorphism associated with increased risk of adverse cardiovascular outcomes: results from GENEtic Substudy of the INternational VErapamil SR-trandolapril STudy (INVEST-GENES). Am Heart J 2008; 156: 397-404.
17. Liu K. et al. Alpha-adducin Gly460Trp polymorphism and hypertension risk: a meta-analysis of 22 studies including 14303 cases and 15961 controls. PLoS One 2010; 5.
18. Niu W. and Qi Y. Association of alpha-adducin and G-protein beta3 genetic polymorphisms with hypertension: a meta-analysis of Chinese populations. PLoS One 2011; 6: e17052.
19. Niu W.Q. et al. Lack of association between alpha-adducin G460W polymorphism and hypertension: evidence from a case-control study and a meta-analysis. J Hum Hypertens 2010; 24: 467-474.
20. Wang R. et al. Association between alpha-adducin gene polymorphism (Gly460Trp) and genetic predisposition to salt sensitivity: a meta-analysis. J Appl Genet 2010; 51: 87-94.
21. Citterio L. et al. Polymorphisms, hypertension and thiazide diuretics. Pharmacogenomics 2011; 12: 1587-

1604.
22. Psaty B.M. et al. Diuretic therapy, the alpha-adducin gene variant, and the risk of myocardial infarction or stroke in persons with treated hypertension. JAMA 2002; 287: 1680-1689.
23. Davis B.R. et al. Antihypertensive therapy, the alpha-adducin polymorphism, and cardiovascular disease in high-risk hypertensive persons: the Genetics of Hypertension-Associated Treatment Study. Pharmacogenomics J 2007; 7: 112-122.
24. Schelleman H. et al. Diuretic-gene interaction and the risk of myocardial infarction and stroke. Pharmacogenomics J 2007; 7: 346-352.
25. Turner S.T. et al. Effects of endothelial nitric oxide synthase, alpha-adducin, and other candidate gene polymorphisms on blood pressure response to hydrochlorothiazide. Am J Hypertens 2003; 16: 834-839.
26. Schelleman H. et al. The influence of the alpha-adducin G460W polymorphism and angiotensinogen M235T polymorphism on antihypertensive medication and blood pressure. Eur J Hum Genet 2006; 14: 860-866.
27. van Wieren-de Wijer D.B. et al. Interaction between the Gly460Trp alpha-adducin gene variant and diuretics on the risk of myocardial infarction. J Hypertens 2009; 27: 61-68.
28. Montalescot G. et al. 2013 ESC guidelines on the management of stable coronary artery disease: the Task Force on the management of stable coronary artery disease of the European Society of Cardiology. Eur Heart J 2013; 34: 2949-3003.
29. Fox K.M. and E.U.t.O.r.o.c.e.w.P.i.s.c.A.d. Investigators. Efficacy of perindopril in reduction of cardiovascular events among patients with stable coronary artery disease: randomised, double-blind, placebo-controlled, multicentre trial (the EUROPA study). Lancet 2003; 362: 782-788.
30. Brugts J.J. et al. The rationale and design of the PERindopril GENEtic association study (PERGENE): a pharmacogenetic analysis of angiotensin-converting enzyme inhibitor therapy in patients with stable coronary artery disease. Cardiovasc Drugs Ther 2009; 23: 171-181.
31. Deckers J.W. et al. Treatment benefit by perindopril in patients with stable coronary artery disease at different levels of risk. Eur Heart J 2006; 27: 796-801.
32. Casari G. et al. Association of the alpha-adducin locus with essential hypertension. Hypertension 1995; 25: 320-326.
33. Nakamura Y. et al. Both angiotensinogen M235T and alpha-adducin G460W polymorphisms are associated with hypertension in the Japanese population. J Hum Hypertens 2007; 21: 253-255.
34. Citterio L. et al. Genetics of primary hypertension: the clinical impact of adducin polymorphisms. Biochim Biophys Acta 2010; 1802: 1285-1298.
35. Cwynar M. et al. Epistatic interaction between alpha- and gamma-adducin influences peripheral and central pulse pressures in white Europeans. J Hypertens 2005; 23: 961-969.
36. 1000 Genomes Project. Available from: https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/.
37. Liao X. et al. Association of alpha-ADD1 Gene and Hypertension Risk: A Meta-Analysis. Med Sci Monit 2015; 21: 1634-1641.
38. Turner S.T. et al. Genomic association analysis of common variants influencing antihypertensive response to hydrochlorothiazide. Hypertension 2013; 62: 391-397.
39. Remme W.J. et al. Secondary prevention of coronary disease with ACE inhibition--does blood pressure reduction with perindopril explain the benefits in EUROPA? Cardiovasc Drugs Ther 2009; 23: 161-170.

13

Haptoglobin polymorphism in relation to coronary plaque characteristics on radiofrequency intravascular ultrasound and near-infrared spectroscopy in patients with coronary artery disease.

13

Haptoglobin polymorphism in relation to coronary plaque characteristics on radiofrequency intravascular ultrasound and near-infrared spectroscopy in patients with coronary artery disease.

Buljubasic N, Oemrawsingh RM, Smeets MB, Cheng JM, Regar E, van Geuns RJ, Serruys PW, Boersma E, Akkerhuis KM, Kardys I, Arslan F.

Int J Cardiol 2016; 15; 221: 682-687. doi: 10.1016/j.ijcard.2016.07.126.

Abstract

Rationale: Conflicting results exist regarding the association between a common Haptoglobin (Hp) polymorphism and risk of coronary artery disease. We investigated the association of three functionally different anti-oxidant and anti-inflammatory Hp phenotypes (Hp1-1, Hp2-1, Hp2-2) with invasively measured degree and composition of coronary atherosclerosis as determined by intravascular ultrasound(-virtual histology) (IVUS(-VH)) as well as near-infrared spectroscopy (NIRS).

Methods & Results: Non-culprit coronary artery segments of 581 patients with acute coronary syndrome (ACS) or stable angina pectoris were imaged with IVUS(-VH). In 203 patients, the segments were also imaged with NIRS. Pre-procedural blood samples were drawn for Hp phenotyping. Degree (segment plaque volume, segment plaque burden (PB); presence of lesions with PB≥70%) and composition (segment fractions of fibrous, fibro-fatty, dense calcium, and necrotic core tissue; presence of IVUS-VH derived thin-cap fibroatheroma lesions) of coronary atherosclerosis were measured. No differences were present between

the three Hp phenotypes with regard to degree and composition of coronary atherosclerosis in the full cohort. However, ACS patients with a Hp2-1 or Hp2-2 phenotype had a higher segment PB percentage (β (95% CI): 3.88 (0.31 – 7.44), p=0.033), increased prevalence of lesions with PB≥70% (OR (95% CI): 3.61 (1.06 – 12.30), p=0.040), and a tendency towards a higher segment plaque volume (β (95% CI): 1.29 (-0.04 – 2.62), p=0.056) in multivariable analyses.

Conclusions: Although in the full cohort no associations could be demonstrated between Hp phenotypes and plaque characteristics, a significant association was present between phenotypes resulting from a genotype containing a Hp2 allele (Hp2-1 or Hp2-2) and a higher degree of atherosclerosis in patients with ACS.

Introduction

Circulating haptoglobin (Hp) is hypothesized to influence atherosclerosis through its anti-oxidant and immunomodulatory properties. Specifically, it prevents hemoglobin-driven oxidative reactions in response to intraplaque hemorrhage, and stimulates a variety of pro- and anti-inflammatory cytokines [1, 2]. The Hp gene carries a common polymorphism with two alleles (Hp1 and Hp2), resulting in three functionally-different phenotypes, each characterized by a unique protein structure: Hp1-1 (wildtype genotype; dimer), Hp2-1 (heterozygous variant; linear polymer) and Hp2-2 (homozygous variant; cyclic polymer) [2]. The homozygous variant Hp2-2 produces a dysfunctional protein with the lowest anti-oxidant and anti-inflammatory properties as compared to the proteins encoded by Hp2-1 or Hp1-1 [1, 2].

Although the molecular functions of these proteins in the vascular wall have been well investigated and seem to be clear, clinical studies on the association of Hp phenotypes with coronary events have rendered conflicting results [3, 4]. In order to further increase understanding of the pathophysiological relation between Hp phenotypes and coronary atherosclerosis, imaging studies using coronary angiography and CT angiography have been performed. However, these have not been able to further elucidate potential mechanisms [5, 6]. The imaging techniques used in these studies only enable evaluation of the lumen of the coronary artery. Conversely, radiofrequency intravascular ultrasound (IVUS) and near-infrared spectroscopy (NIRS) enable evaluation and quantification of the arterial wall itself.

However, studies on Hp phenotype and invasively-measured coronary atherosclerotic plaque characteristics by IVUS or NIRS are currently lacking.

In the current study, we investigated the relation between Hp phenotype and in-vivo measurements of degree and composition of coronary atherosclerosis by IVUS and NIRS in 581 patients undergoing coronary angiography. Herewith, we aimed to provide additional insights into the pathophysiology concerning Hp and coronary atherosclerosis.

Methods

The rationale and design of the ATHEROREMO-IVUS study and its ATHEROREMO-NIRS substudy have been described in detail elsewhere [7-9]. These studies were approved by the medical ethics committee of the Erasmus MC and performed in accordance with the declaration of Helsinki. All included patients provided written informed consent.

In brief, 581 patients with an indication for coronary angiography due to stable angina pectoris (SAP) or acute coronary syndrome (ACS) underwent IVUS imaging of a non-stenotic segment of at least 40mm in length in a predefined non-culprit coronary artery with the Volcano™ s5/s5i Imaging System (Volcano Corp., San Diego, USA), using the Volcano™ Eagle Eye Gold IVUS catheter (20MHz) [7]. The order of preference for selection of the non-culprit coronary artery segment was: 1. Left anterior descending artery; 2. Right coronary artery; 3. Left circumflex artery. An automatic pullback system was used with a standard pull back speed of 0.5mm per second. Both IVUS grayscale and virtual histology (IVUS-VH) analyses were performed using pcVH 2.1 and qVH (Volcano Corp., San Diego, USA) software. The external elastic membrane and luminal borders were contoured for each frame (median interslice distance, 0.40mm). The degree and composition of each atherosclerotic plaque were assessed. Plaque volume (mm^3) was defined as the total volume of the external elastic membrane occupied by atheroma and normalized for the length of the imaged segment. Plaque burden (%) was defined as plaque and media cross-sectional area divided by external elastic membrane cross-sectional area and is presented as a percentage. Atherosclerotic plaque composition was characterized into fibrous (FI), fibro-fatty (FF), dense calcium (DC) and necrotic core (NC) tissue and expressed as percentages of total plaque volume. Three types of high-risk lesions were identified: 1. Virtual Histology-IVUS derived thin-cap fibroatheroma (VH-TCFA) lesions (presence of >10% confluent necrotic

core in direct contact with the lumen); 2. Lesions with plaque burden ≥70%; 3. Lesions with a minimal luminal area ≤4.0 mm^2. Hp phenotypes were successfully determined in 574 of the patients who underwent IVUS(-VH) imaging (Figure 1). NIRS (InfraReDx, Burlington, Massachusetts, USA) of the same segment was performed in a subset of 191 patients, as well as 12 additional patients that only underwent NIRS, not IVUS [7, 9] (Figure 1). The U.S. Food and Drug Administration-approved NIRS system, as used in this study, consisted of a 3.2-F rapid exchange catheter, a pullback and rotation device. Image acquisition was performed by a motorized catheter pullback at a speed of 0.5mm per second and 240 rotations per minute. The Lipid Core Burden Index (LCBI) score was measured and represents the amount of lipid core in the imaged segment on a 0-to-1000 scale, as described previously [9]. Both IVUS and NIRS images were analyzed off-line by an independent core laboratory (Cardialysis BV, Rotterdam, the Netherlands), by personnel blinded for baseline patient characteristics and Hp phenotypes. Two-hundred of the 203 patients who underwent NIRS imaging, were successfully phenotyped, resulting in a total of 585 successfully phenotyped and imaged patients for the current investigation (Figure 1).

EDTA plasma samples were drawn from the arterial sheath prior to coronary angiography and transported to the clinical laboratory of the Erasmus MC for further processing and storage at a temperature of -80°C within two hours after blood collection. After completion of the cohort, all frozen EDTA-plasma samples were transported under controlled conditions (at a temperature of -80°C) to the Laboratory of Experimental Cardiology, University Medical Center Utrecht, Utrecht, The Netherlands, where Hp phenotypes were differentiated through western blotting.

The primary endpoint consisted of major adverse cardiovascular events (MACE), and was defined as a composite of all-cause mortality, ACS, and unplanned coronary revascularization. The secondary endpoint consisted of the composite of all-cause mortality and ACS. ACS was defined as the clinical diagnosis of (non-)ST-segment elevation myocardial infarction or unstable angina pectoris [10, 11]. Unplanned coronary revascularization was defined as any repeat PCI or coronary artery bypass grafting that was not foreseen at the index procedure. Follow-up data were collected during 1 year. Vital status was obtained from municipal civil registries and questionnaires were sent focusing on the occurrence of MACE to all living patients (response rate of 92.3%). Upon patients' approval, additional information was obtained from hospital discharge letters and treating physicians whenever necessary. Endpoints were adjudicated based on original source data by a clinical events committee.

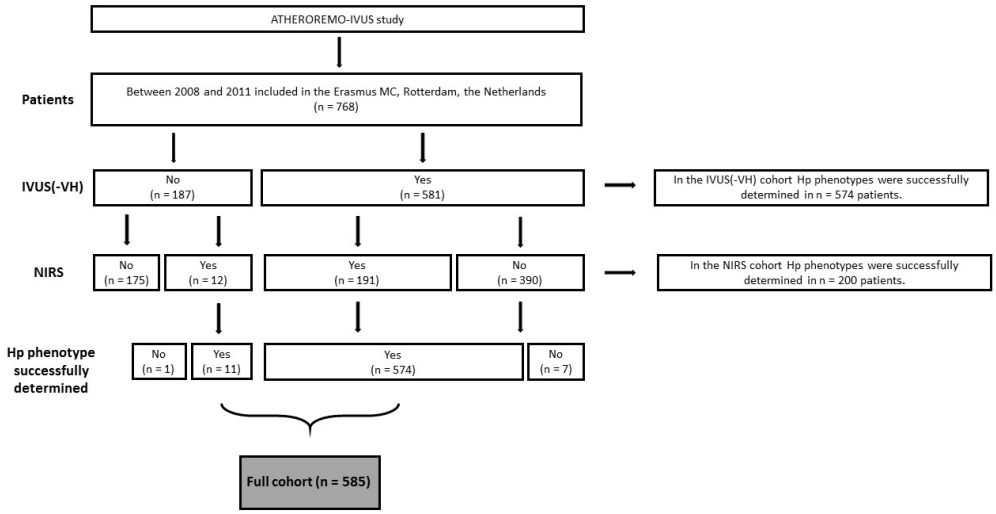


Figure 1. Flowchart patient inclusion in the ATHEROREMO-IVUS study, ATHEROREMO-NIRS substudy and ATHEROREMO Haptoglobin phenotype substudy.

ACS=acute coronary syndrome; Hp=Haptoglobin; IVUS(-VH)=intravascular ultrasound(-virtual histology); NIRS=near-infrared spectroscopy.

Variables with a non-normal distribution were transformed by using either the natural logarithm (ln) or square root for further analyses. Univariate analyses were performed by ANOVA or Student's t-test for continuous variables and Chi-squared test for categorical variables, comparing the phenotypes. Multivariate linear and logistic regression analyses were performed with Hp phenotype as the independent variable and with adjustment for the potential confounders age, gender, smoking, diabetes mellitus, hypertension, dyslipidemia and previous myocardial infarction. Interaction terms were used to test for effect modification by indication for angiography (ACS versus SAP). Subsequently, analyses were stratified on indication. Since ACS subgroup analysis showed (VH-)IVUS values of similar magnitude in the Hp2-1 and Hp2-2 groups, a post-hoc analysis was performed comparing ACS patients with wildtype Hp1-1 versus a pooled group with the variant phenotypes Hp2-1 and Hp2-2. Furthermore, a subgroup analysis was performed in diabetic patients (n=99). Finally, the association between Hp phenotype and clinical endpoints after 1 year of follow-up was examined with Cox proportional hazard regression analyses.

All data were analyzed with SPSS software (IBM SPSS Statistics for Windows, Version 23, Armonk, NY, USA). All statistical tests were two-tailed and p-values <0.05 were considered statistically significant.

Results

Baseline characteristics

Baseline clinical and procedural characteristics of the 3 phenotype groups are presented in Table 1. Prevalence of phenotype Hp1-1, Hp2-1 and Hp2-2 was 16.1% (n=94), 45.5% (n=266), and 38.5% (n=225), respectively, and this distribution was in Hardy-Weinberg equilibrium (p=0.67). Mean age±standard deviation was 61.9±12.6, 62.4±10.7 and 60.0±11.4 years, respectively (p=0.07). Except for history of myocardial infarction, there were no differences in clinical or procedural characteristics across the various phenotypes. As expected, circulating plasma haptoglobin concentration was lowest for Hp1-1 and highest for Hp2-2 (p=<0.001, Table 1).

Table 1. Baseline clinical and procedural characteristics of the Haptoglobin phenotype groups in the full cohort (n = 585).

	Haptoglobin 1-1 (n = 94)	Haptoglobin 1-2 (n = 266)	Haptoglobin 2-2 (n = 225)	P-value[a]
Clinical characteristics				
Age, years	61.9 ± 12.6	62.4 ± 10.7	60.0 ± 11.4	0.07
Male gender, n (%)	69 (73.4)	200 (75.2)	173 (76.9)	0.79
Diabetes mellitus, n (%)	11 (11.7)	46 (17.3)	43 (19.1)	0.28
Hypertension, n (%)	48 (51.1)	138 (51.9)	118 (52.7)	0.96
Dyslipidemia, n (%)	45 (47.9)	154 (57.9)	126 (56.3)	0.24
Smoking, n (%)	25 (26.6)	83 (31.2)	63 (28.0)	0.61
Positive family history, n (%)	58 (61.7)	139 (52.3)	110 (49.1)	0.12
Peripheral artery disease, n (%)	4 (4.3)	18 (6.8)	14 (6.2)	0.68
Previous myocardial infarction, n (%)	24 (25.5)	98 (36.8)	64 (28.4)	0.050
Previous PCI, n (%)	26 (27.7)	91 (34.2)	73 (32.4)	0.51
Previous CABG, n (%)	3 (3.2)	9 (3.4)	7 (3.1)	0.99
Previous stroke, n(%)	3 (3.2)	12 (4.5)	10 (4.4)	0.85
History of renal insufficiency, n (%)	4 (4.3)	21 (7.9)	8 (3.6)	0.10
Haptoglobin level, mg/ml	0.79 [0.58 – 0.99]	1.53 [1.10 – 2.20]	1.60 [1.10 – 2.30]	<0.001
Procedural characteristics				
Indication for coronary angiography				
Acute coronary syndrome, n (%)	49 (52.1)	144 (54.1)	127 (56.4)	0.76
Stable angina pectoris, n (%)	45 (47.9)	122 (45.9)	98 (43.6)	0.76
Coronary artery disease				
No significant stenosis, n (%)	4 (4.3)	17 (6.4)	22 (9.8)	0.16
1-vessel disease, n (%)	57 (60.6)	138 (51.9)	117 (52.0)	0.30
2-vessel disease, n (%)	26 (27.7)	82 (30.8)	60 (26.7)	0.58
3-vessel disease, n (%)	7 (7.4)	29 (10.9)	26 (11.6)	0.54
PCI performed	86 (91.5)	233 (87.6)	195 (86.7)	0.48

PCI=percutaneous coronary intervention; CABG=coronary artery bypass graft surgery. Continuous variables are presented as mean ± SD or median [interquartile range], depending on their distribution. Categorical variables are presented as n (%). [a]P-values obtained by ANOVA for the continuous variables and Chi-squared test for the categorical variables.

Degree and composition of coronary atherosclerosis

No differences could be demonstrated between the different phenotypes with regard to the degree and composition of atherosclerosis as assessed by IVUS-VH or NIRS (Table 2). The same could be concluded for the subgroup analysis of diabetic patients (n=99, data not shown).

Table 2. NIRS and (VH-)IVUS segment and lesion characteristics of the Haptoglobin phenotype groups in the full cohort (n = 585).

	Haptoglobin 1-1 (n = 94)	Haptoglobin 2-1 (n = 266)	Haptoglobin 2-2 (n = 225)	P-value[c]
Segment plaque characteristics[a]				
Degree of atherosclerosis				
Plaque volume, mm^3	240.7 [118.5 – 313.4]	235.1 [150.8 – 332.9]	216.0 [147.6 – 323.3]	0.94
Plaque burden, %	37.4 ± 12.1	38.3 ± 11.6	38.4 ± 11.1	0.80
Plaque composition				
Fibrous percentage	57.6 ± 12.0	57.4 ± 11.4	58.2 ± 11.7	0.74
Fibro-fatty percentage	9.1 [5.9 – 12.4]	9.2 [5.9 – 13.3]	8.4 [5.3 – 11.9]	0.19
Necrotic core percentage	21.7 ± 8.1	21.0 ± 8.3	21.8 ± 7.7	0.57
Dense calcium percentage	9.5 [5.3 – 14.4]	9.5 [5.4 – 15.3]	9.1 [4.9 – 15.1]	0.92
Lipid Core Burden Index (LCBI)[b]	47.5 [9.0 – 93.5]	40.5 [16.0 – 85.8]	40.0 [13.3 – 80.8]	0.82
Lesion plaque characteristics[a]				
Degree of atherosclerosis				
≥1 Lesion with PB ≥70%, n (%)	18 (20.2)	54 (20.5)	51 (23.2)	0.74
≥1 Lesion with MLA ≤4.0mm^2, n (%)	29 (32.6)	83 (31.7)	68 (30.9)	0.96
Plaque composition				
≥1 TCFA, n (%)	43 (48.3)	106 (40.3)	91 (41.2)	0.40

PB=plaque burden; MLA=minimal lumen area; TCFA=thin-cap fibroatheroma. Continuous variables are presented as mean ± SD or median [interquartile range], depending on their distribution. Categorical variables are presented as n (%). [a]IVUS-VH imaging was performed in 574 patients. [b]NIRS imaging was performed in a subset of 200 patients. [c]P-values obtained by ANOVA for the continuous variables and Chi-squared test for the categorical variables.

Significant interactions were present between Hp phenotype and indication for angiography (ACS versus SAP) for the association with plaque volume, plaque burden, FI tissue percentage and lesions with PB≥70% (p-values for interaction all <0.05 in uni- and multivariate analysis). In line with this, in ACS patients, phenotypes resulting from a genotype containing a Hp2 allele (Hp2-1 or Hp2-2) were significantly associated with a higher plaque volume (p=0.031) in univariate analysis and tended to be associated with a higher plaque volume in multivariate analysis (β (95% CI): 1.29 (-0.04 – 2.62) mm^3 increase in (square root transformed) plaque volume for having Hp2-1 or Hp2-2 as compared to Hp1-1, p=0.056) (Table 3, Figure 2). Moreover, in ACS patients these phenotypes were independently associated with a larger

plaque burden (β (95% CI): 3.88 (0.31 – 7.44) % increase in PB for having Hp2-1 or Hp2-2 as compared to Hp1-1, p=0.033) (Table 3, Figure 3), as well as an increased prevalence of lesions with PB≥70% (OR (95%CI): 3.61 (1.06 – 12.30), p=0.040) (Table 3, Figure 4). With respect to atherosclerotic plaque composition, no associations were present with the various VH-tissue types, LCBI or VH-TCFA lesions in ACS patients (Table 3).

Table 3. NIRS and (VH)-IVUS segment and lesion characteristics of the Haptoglobin phenotype groups in patients with ACS (n = 320).

	Haptoglobin 1-1 (n = 49)	**Haptoglobin 2-1 or 2-2 (n = 271)**	**P-value[c]**	**β (95% CI)**	**P-value[d]**
Haptoglobin level, mg/ml	0.84 [0.63 – 1.10]	1.60 [1.10 – 2.50]	<0.001	0.65 (0.45 – 0.85)	<0.001
Segment plaque characteristics[a]					
Degree of atherosclerosis					
Plaque volume, mm^3	173.5 [107.3 – 303.1]	215.5 [141.2 – 304.2]	0.031	1.29 (-0.04 – 2.62)	0.056
Plaque burden, %	32.9 ± 10.6	37.6 ± 11.8	0.014	3.88 (0.31 – 7.44)	0.033
Plaque composition					
Fibrous percentage	61.4 ± 11.2	58.5 ± 12.0	0.13	-2.99 (-6.80 – 0.83)	0.12
Fibro-fatty percentage	8.6 [4.6 – 12.0]	8.6 [5.5 – 12.0]	0.35	0.18 (-0.14 – 0.51)	0.26
Necrotic core percentage	21.2 ± 8.3	21.8 ± 8.6	0.68	0.43 (-2.37 – 3.22)	0.76
Dense calcium percentage	6.7 [4.9 – 11.3]	8.3 [4.9 – 13.8]	0.28	0.21 (-0.17 – 0.58)	0.28
Lipid Core Burden Index (LCBI)[b]	48.0 [6.0 – 91.0]	44.5 [16.0 – 88.0]	0.53	0.03 (-0.71 – 0.77)	0.93
Lesion plaque characteristics[a]			**P-value[c]**	**OR (95% CI)**	**P-value[d]**
Degree of atherosclerosis					
≥1 Lesion with PB≥70%, n (%)	3 (6.7)	56 (21.0)	0.023	3.61 (1.06 – 12.30)	0.040
≥1 Lesion with MLA ≤4.0mm^2, n (%)	11 (24.4)	80 (30.1)	0.44	1.30 (0.61 – 2.70)	0.51
Plaque composition					
≥1 TCFA, n (%)	22 (48.9)	119 (44.6)	0.59	0.78 (0.41 – 1.51)	0.46

Continuous variables are presented as mean ± SD or median [interquartile range], depending on the distribution. Categorical variables are presented as n (%). Beta (β) indicates the increase or decrease (minus sign) in each (transformed) imaging segment parameter for the Haptoglobin 2-1 or 2-2 ACS patients as compared to Haptoglobin 1-1 ACS patients. Odds ratio (OR) increase in each lesion parameter for the Haptoglobin 2-1 or 2-2 ACS patients as compared to Haptoglobin 1-1 ACS patients. [a]IVUS-VH imaging was performed in 313 ACS patients. [b]NIRS imaging was performed in a subset of 93 ACS patients. [c]P-values (univariate) obtained by the independent Student's two-sample t-test for the continuous variables and Chi-squared test for the categorical variables. [d]P-values (multivariate) obtained by linear regression analyses for continuous variables and logistic regression analyses for categorical variables with Haptoglobin 1-1 as the reference category. Models adjusted for age, gender, smoking, diabetes mellitus, hypertension, dyslipidemia and previous myocardial infarction.

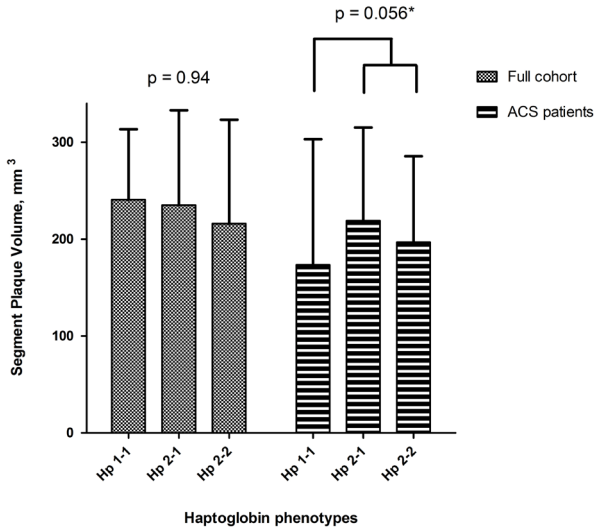


Figure 2. Segment plaque volume in the Haptoglobin phenotypes within the full cohort and within the ACS subgroup.

ACS=acute coronary syndrome; Hp=Haptoglobin.

* P-value for difference in segment plaque volume between Hp2-1 or Hp2-2 as compared to Hp1-1 within the ACS subgroup. Adjusted for age, gender, smoking, diabetes mellitus, hypertension, dyslipidemia and previous myocardial infarction.

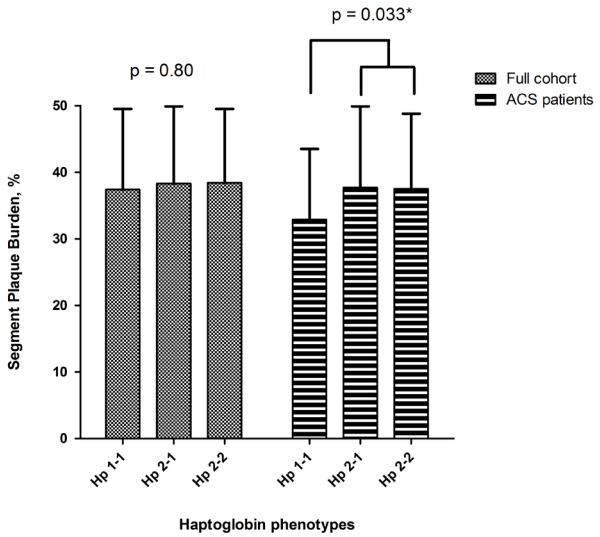


Figure 3. Segment plaque burden in the Haptoglobin phenotypes within the full cohort and ACS subgroup.

ACS=acute coronary syndrome; Hp=Haptoglobin.

* P-value for difference in segment plaque burden between Hp2-1 or Hp2-2 as compared to Hp1-1 within the ACS subgroup. Adjusted for age, gender, smoking, diabetes mellitus, hypertension, dyslipidemia and previous myocardial infarction.

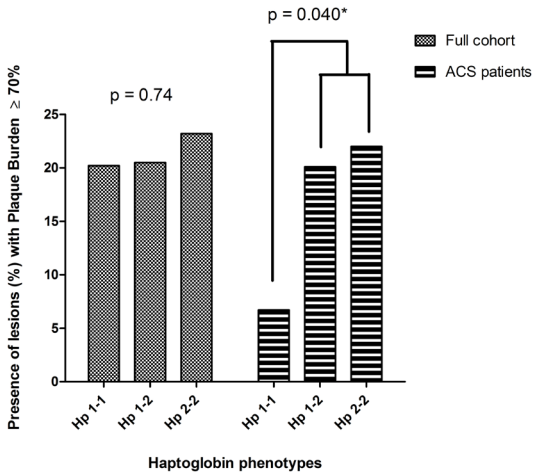


Figure 4. Presence of large lesions in the Haptoglobin phenotypes within the full cohort and ACS subgroup.

ACS=acute coronary syndrome; Hp=Haptoglobin.

* P-value for difference in presence of large lesions between Hp2-1 or Hp2-2 as compared to Hp1-1 within the ACS subgroup. Adjusted for age, gender, smoking, diabetes mellitus, hypertension, dyslipidemia and previous myocardial infarction.

Clinical endpoints

With regard to clinical outcome, associations between Hp phenotype and 1-year cardiovascular outcome could not be demonstrated, both in the full cohort and in the ACS and diabetes subgroups. In particular, the Hp phenotypes were not associated with the occurrence of MACE (primary composite endpoint) on multivariate analysis: HR (95% CI) 0.88 (0.52 – 1.49) for Hp2-1 and 0.97 (0.57 – 1.67) for Hp2-2 in the full cohort; HR (95% CI) 0.77 (0.38 – 1.56) for Hp2-1 and 0.70 (0.33 – 1.49) for Hp2-2 in the ACS subgroup; HR (95% CI) 0.91 (0.30 – 2.82) for Hp2-1 and 0.94 (0.30 – 3.02) for Hp2-2 in the diabetic subgroup.

Discussion

To our knowledge, this is the first study that investigated the relation between Hp phenotypes and coronary plaque characteristics as assessed with IVUS-VH and NIRS in patients with CAD. Although no associations could be demonstrated between Hp phenotypes and coronary plaque characteristics in the full cohort, in ACS patients phenotypes Hp2-1 and Hp2-2 were significantly associated with a higher degree of coronary atherosclerosis as expressed by higher segment plaque burden and higher prevalence of lesions with PB≥70%, as compared to Hp1-1.

Existing imaging studies on Hp phenotype and atherosclerosis as assessed with either coronary angiography or CT angiography are limited in number and were mainly performed in specifically defined study populations, such as patients with diabetes mellitus [5, 6, 12, 13]. Overall, these studies did not find any associations between Hp phenotype and coronary atherosclerosis [5, 6, 12, 13]. The interaction between Hp phenotype and acute versus stable clinical presentation of CAD has not been investigated earlier. A potential, biologically plausible explanation for this interaction could be that patients who ultimately experience ACS represent a subgroup that exhibits an increased pro-inflammatory [14] and oxidative state [15-17] as compared to SAP patients. In these patients, elevated oxidative stress may not only be present systemically [15, 16] but also locally at the level of the atherosclerotic plaque, as part of the pathogenesis and evolution towards an ACS. The latter results, among others, from intraplaque hemorrhage, which occurs more often in ACS than in SAP patients, and gives rise to a local release of hemoglobin (iron) into the atherosclerotic plaque [18]. Such a state leads, among several other reactions, to local generation of reactive oxygen species and consequently lipid peroxidation [15], which eventually may contribute to accelerated atherosclerotic plaque growth [15, 19] in these patients. This process may be further enhanced in Hp2 phenotypes (Hp2-1 and Hp2-2) due to their reduced anti-oxidant and anti-inflammatory properties as compared to Hp1-1 [1, 2]. A previous study in mice supports this hypothesis by demonstrating increased iron, lipid peroxidation and macrophage accumulation in Hp2-2 atherosclerotic plaques as compared with Hp1-1 plaques [20]. This was confirmed in humans by autopsy studies that have demonstrated more advanced atherosclerotic plaques in Hp2-2 compared to Hp1-1 individuals [21]. In contrast to Hp1-1 proteins, the Hp2-1 and Hp 2-2 proteins have low affinity for both hemoglobin and the macrophage CD163 scavenger receptor in order to clear hemoglobin (iron) from the atherosclerotic plaque and prevent its

harmful intraplaque oxidative reactions [2, 22]. Altogether, these studies indicate that oxidative stress might strongly be implicated in the atherosclerotic process with a critical role for Hp proteins in its further development.

In a previous study within the same cohort, we could not demonstrate an association between plasma Hp concentration and (VH-)IVUS plaque characteristics or clinical events [8]. Although the biological function of Hp in the vascular wall might not directly depend on its plasma concentrations, but rather on its protein structure, there is a direct correlation of Hp phenotype with Hp plasma concentrations. Specifically, Hp concentration is higher in Hp2-2 than in Hp1-1 individuals, because of the weaker binding of Hp2-2 proteins to hemoglobin and the macrophage CD163 receptor [22]. Thus, since Hp concentrations may at least in part be phenotype-dependent, these negative results seem consistent with our current findings.

Epidemiologic studies investigating the association between Hp phenotype and incidence of CAD in the general population are limited in number and have yielded contradicting results. While De Baquer et al found that Hp1-1 individuals were at higher risk of CAD mortality as compared to the other Hp phenotypes [3], the Framingham Heart Offspring Study (n=3273) could not demonstrate any relationship between Hp phenotype and CAD prevalence in the overall study population [4].

The majority of clinical studies concerning Hp phenotypes has focused on diabetic individuals, since strong evidence exists that Hp phenotype and diabetic state significantly interact with regard to prevalence of CAD. It has been demonstrated that Hp2-2 individuals with diabetes have a higher risk of adverse cardiovascular outcomes as compared to the other phenotypes [4, 23-27], which is thought to be caused by the decreased anti-oxidant capabilities of the Hp2-2 protein in conjunction with an exceptionally high level of oxidative stress in diabetes [28, 29]. However, some other studies could not confirm these findings [30], or even rendered contradictory results [4]. We also could not demonstrate any associations with in-vivo coronary plaque characteristics or 1-year clinical outcome in diabetic patients. Our findings are in agreement with a study in type 2 diabetic patients, that could not demonstrate an association between Hp genotype and coronary artery calcification (CAC) as a reflection of total coronary atherosclerotic burden [5]. On the other hand, a larger case-control study on type 1 diabetic patients found that the Hp2-2 genotype was a predictor of CAC progression. The limited number of diabetic participants (n=99) in our cohort may have contributed to the lack of such an association in our study.

Our study has several limitations that warrant acknowledgement. Firstly, our findings in

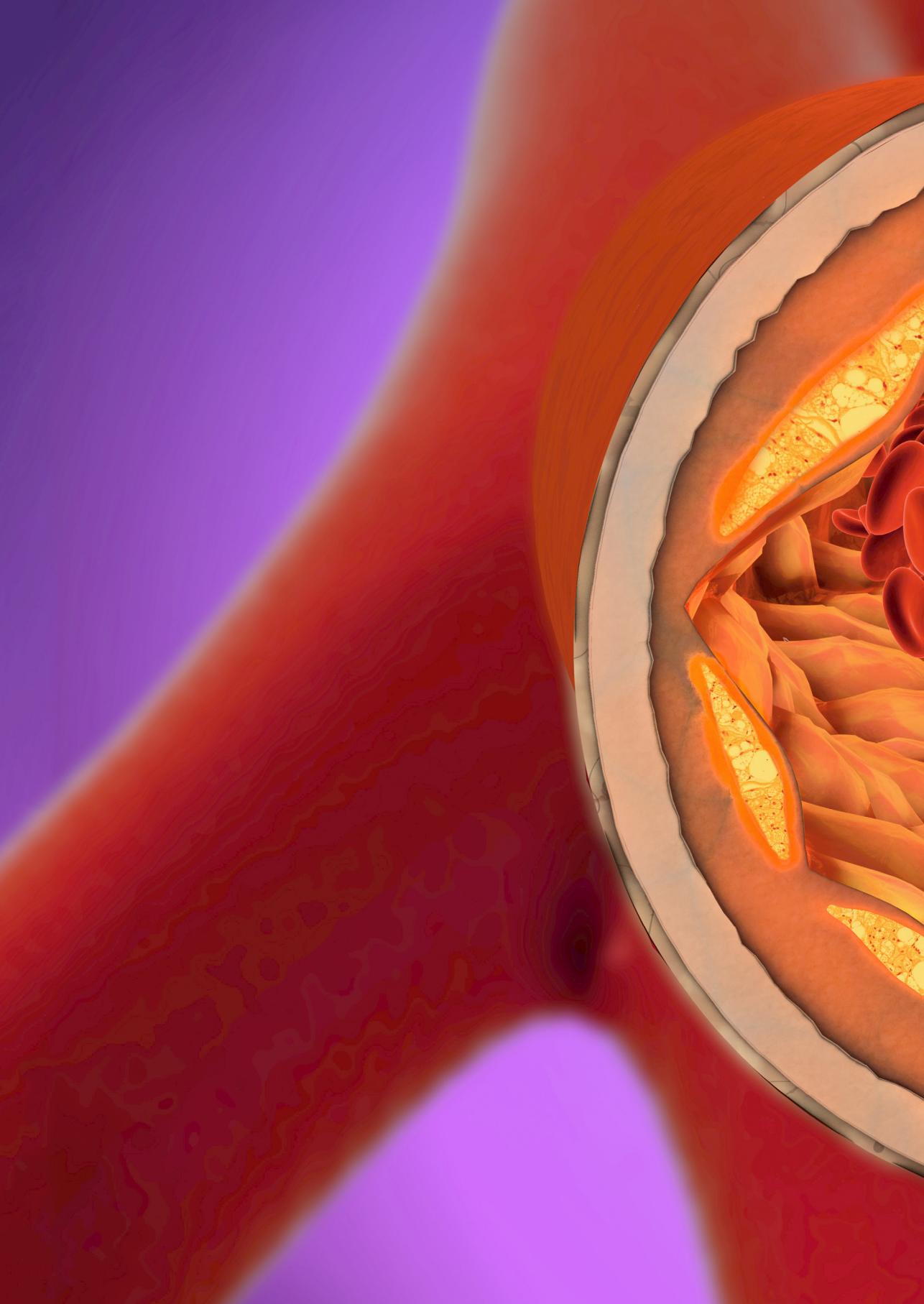
the ACS subgroup should be considered as hypothesis-generating, because the comparison of Hp2-2 and Hp2-1 on the one hand with Hp1-1 on the other hand in the ACS patients was a post-hoc analysis. Nevertheless, the interaction terms between Hp phenotypes and indication for catheterization were highly significant in multivariate analyses. Secondly, IVUS(-VH) imaging took place of a non-culprit coronary artery segment only. However, this approach was developed under the hypothesis that such a non-culprit target segment adequately reflects coronary wall pathophysiology of the larger coronary tree, and this hypothesis has been confirmed by several studies [31, 32]. Finally, this study was not primarily designed to investigate the association between Hp phenotypes and atherosclerosis and clinical outcome in diabetic patients. A small number of diabetic patients in this study may have contributed to the lack of significant associations between Hp phenotypes and degree and composition of atherosclerosis in this subgroup.

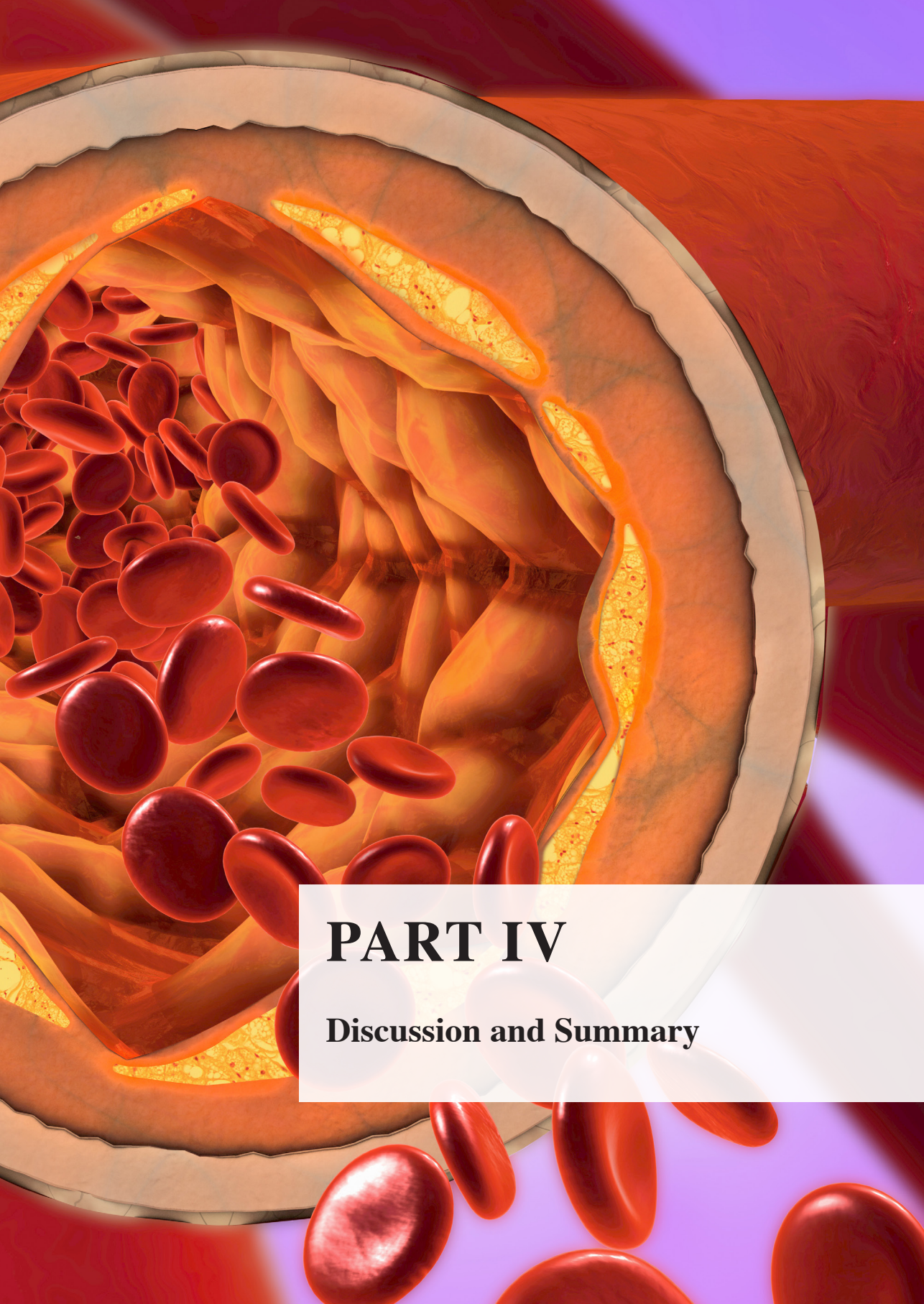
In conclusion, in patients undergoing coronary angiography, no associations were present between Hp phenotypes and invasively-measured coronary atherosclerotic plaque characteristics by IVUS and NIRS. However, patients with Hp2-1 or Hp2-2 presenting with ACS had a significantly higher degree of coronary atherosclerosis as compared to Hp1-1. Thus, genetic differences in the endogenous anti-oxidant status, as reflected by the haptoglobin phenotype, may be of considerable importance in patients suffering from CAD. Our hypothesis-generating findings should be confirmed by other, large studies in order to identify patient groups that might benefit from risk stratification by Hp phenotyping in the future.

References

1. Guetta J. et al. Haptoglobin genotype modulates the balance of Th1/Th2 cytokines produced by macrophages exposed to free hemoglobin. Atherosclerosis 2007; 191: 48-53.
2. Melamed-Frank M. et al. Structure-function analysis of the antioxidant properties of haptoglobin. Blood 2001; 98: 3693-3698.
3. De Bacquer D. et al. Haptoglobin polymorphism as a risk factor for coronary heart disease mortality. Atherosclerosis 2001; 157: 161-166.
4. Levy A.P. et al. Haptoglobin phenotype and prevalent coronary heart disease in the Framingham offspring cohort. Atherosclerosis 2004; 172: 361-365.
5. Jaffe R. et al. Haptoglobin genotype does not predict extent of coronary artery calcification in a prospective cohort of patients with type 2 diabetes. Int J Cardiol 2014; 171: 307-308.
6. Moussa A. et al. Association between haptoglobin 2-2 genotype and coronary artery disease and its severity in a tunisian population. Biochem Genet 2014; 52: 269-282.
7. de Boer S.P. et al. Relation of genetic profile and novel circulating biomarkers with coronary plaque phenotype as determined by intravascular ultrasound: rationale and design of the ATHEROREMO-IVUS study. EuroIntervention 2014; 10: 953-960.
8. Battes L.C. et al. Circulating acute phase proteins in relation to extent and composition of coronary atherosclerosis and cardiovascular outcome: results from the ATHEROREMO-IVUS study. Int J Cardiol 2014; 177: 847-853.
9. Oemrawsingh R.M. et al. Near-infrared spectroscopy predicts cardiovascular outcome in patients with coronary artery disease. J Am Coll Cardiol 2014; 64: 2510-2518.
10. Van de Werf F. et al. Management of acute myocardial infarction in patients presenting with persistent ST-segment elevation: the Task Force on the Management of ST-Segment Elevation Acute Myocardial Infarction of the European Society of Cardiology. Eur Heart J 2008; 29: 2909-2945.
11. Hamm C.W. et al. ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: The Task Force for the management of acute coronary syndromes (ACS) in patients presenting without persistent ST-segment elevation of the European Society of Cardiology (ESC). Eur Heart J 2011; 32: 2999-3054.
12. Simpson M. et al. Haptoglobin genotype predicts development of coronary artery calcification in a prospective cohort of patients with type 1 diabetes. Cardiovasc Diabetol 2011; 10: 99.
13. Lee C.W. et al. Plasma haptoglobin concentrations are elevated in patients with coronary artery disease. PLoS One 2013; 8: e76817. doi: 10.1371/journal.pone.0076817.
14. Libby P. et al. Inflammation and atherosclerosis. Circulation 2002; 105: 1135-1143.
15. Ehara S. et al. Elevated levels of oxidized low density lipoprotein show a positive relationship with the severity of acute coronary syndromes. Circulation 2001; 103: 1955-1960.
16. Patel P.J. et al. The anti-oxidative capacity of high-density lipoprotein is reduced in acute coronary syndrome but not in stable coronary artery disease. J Am Coll Cardiol 2011; 58: 2068-2075.
17. Lorgis L. et al. The free oxygen radicals test (FORT) to assess circulating oxidative stress in patients with acute myocardial infarction. Atherosclerosis 2010; 213: 616-621.
18. Jeney V. et al. Red blood cell, hemoglobin and heme in the progression of atherosclerosis. Front Physiol 2014; 5: 379.
19. Daemen M.J. and Kooi M.E. Intraplaque hemorrhage as a stimulator of episodic growth of advanced, but

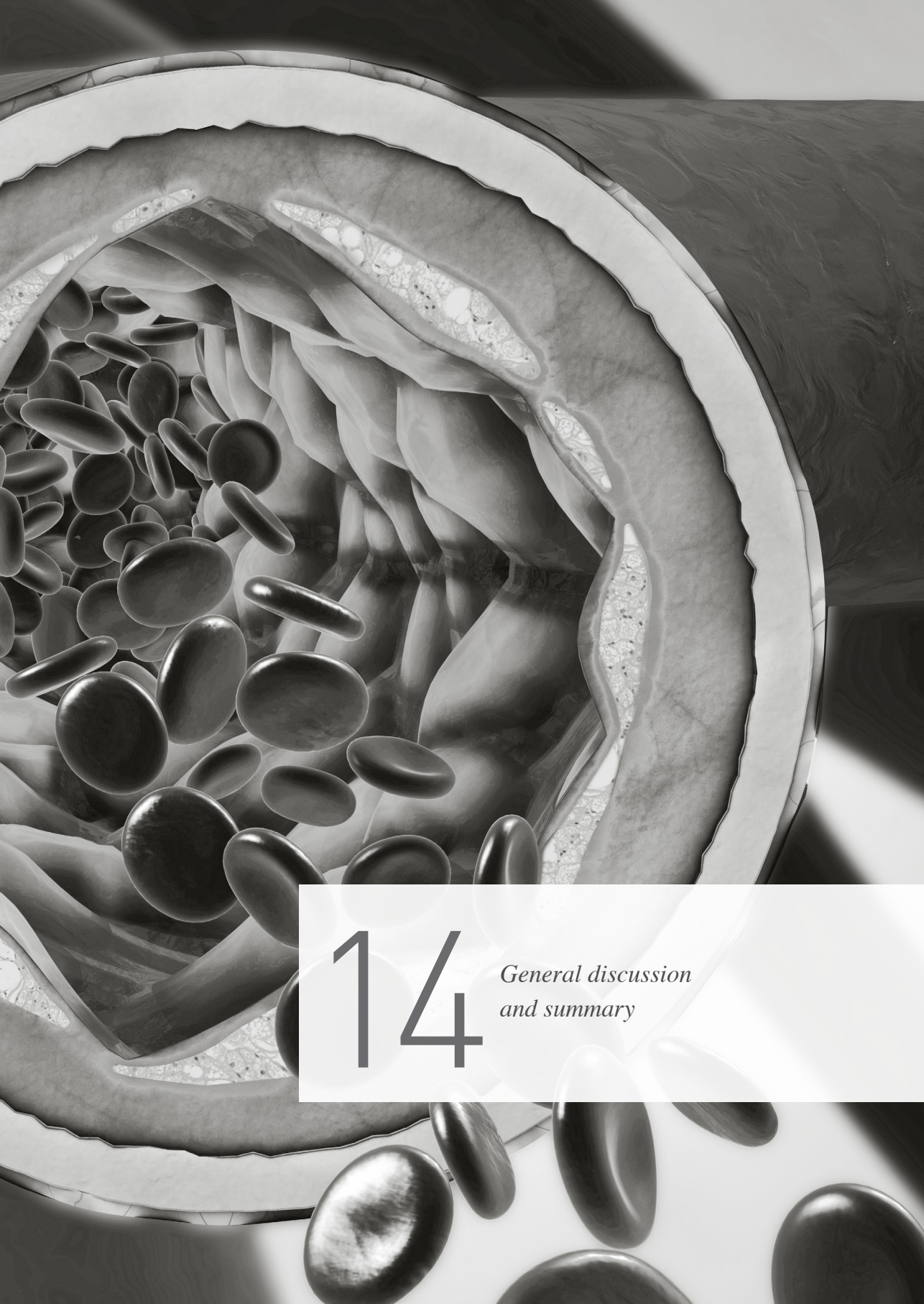
nonsymptomatic atherosclerotic lesions bridging the gap. JACC Cardiovasc Imaging 2009; 2: 1390-1392.
20. Levy A.P. et al. Haptoglobin genotype is a determinant of iron, lipid peroxidation, and macrophage accumulation in the atherosclerotic plaque. Arterioscler Thromb Vasc Biol 2007; 27: 134-140.
21. Moreno P.R. et al. Haptoglobin genotype is a major determinant of the amount of iron in the human atherosclerotic plaque. J Am Coll Cardiol 2008; 52: 1049-1051.
22. Langlois M.R. and Delanghe J.R.. Biological and clinical significance of haptoglobin polymorphism in humans. Clin Chem 1996; 42: 1589-1600.
23. Levy A.P. et al. Haptoglobin phenotype is an independent risk factor for cardiovascular disease in individuals with diabetes: The strong heart study. J Am Coll Cardiol 2002; 40: 1984-1990.
24. Suleiman M. et al. Haptoglobin polymorphism predicts 30-day mortality and heart failure in patients with diabetes and acute myocardial infarction. Diabetes 2005; 54: 2802-2806.
25. Asleh R. and Levy A.P. In vivo and in vitro studies establishing haptoglobin as a major susceptibility gene for diabetic vascular disease. Vasc Health Risk Manag 2005; 1: 19-28.
26. Cahill L.E. et al. Haptoglobin genotype is a consistent marker of coronary heart disease risk among individuals with elevated glycosylated hemoglobin. J Am Coll Cardiol 2013; 61: 728-737.
27. Cahill L.E. et al. The Risk of Coronary Heart Disease Associated With Glycosylated Hemoglobin of 6.5% or Greater Is Pronounced in the Haptoglobin 2-2 Genotype. J Am Coll Cardiol 2015; 66: 1791-1799.
28. Awadallah S.M. et al. Haptoglobin polymorphism in relation to antioxidative enzymes activity in type 2 diabetes mellitus. Diabetes Metab Syndr 2013; 7: 26-31.
29. Levy A.P. et al. Downregulation of the hemoglobin scavenger receptor in individuals with diabetes and the Hp 2-2 genotype: implications for the response to intraplaque hemorrhage and plaque vulnerability. Circ Res 2007; 101: 106-110.
30. Pechlaner R. et al. Haptoglobin 2-2 genotype is not associated with cardiovascular risk in subjects with elevated glycohemoglobin-results from the Bruneck Study. J Am Heart Assoc 2014; 3: e000732. doi: 10.1161/JAHA.113.000732.
31. Cheng J.M. et al. In vivo detection of high-risk coronary plaques by radiofrequency intravascular ultrasound and cardiovascular outcome: results of the ATHEROREMO-IVUS study. Eur Heart J 2014; 35: 639-647.
32. Nicholls S.J. et al. Intravascular ultrasound-derived measures of coronary atherosclerotic plaque burden and clinical outcome. J Am Coll Cardiol 2010; 55: 2399-2407.





PART IV

Discussion and Summary



14 *General discussion and summary*

14 General discussion and summary

Recurrent adverse cardiovascular events still occur in a substantial proportion of coronary artery disease (CAD) patients, despite enhanced technology and augmented treatment options. The need for improved risk prediction in patients with CAD has therefore been the main drive for this thesis. By expanding our knowledge on blood biomarkers and genetic polymorphisms that may play a part in the pathophysiology of CAD, a road to the future of optimal risk stratification in patients with established CAD is created. In the present chapter, the main findings of this thesis are summarized and placed into a broader context.

1. Better understanding of human coronary atherosclerosis by linking blood biomarkers to intracoronary imaging.

In the first part of this thesis, biological processes that might be linked to atherosclerosis are studied in relation to coronary atherosclerosis by means of intracoronary imaging within the *European Collaborative Project on Inflammation and Vascular Wall Remodeling in Atherosclerosis – Intravascular Ultrasound (ATHEROREMO-IVUS) study*. The characteristics of each atherosclerotic plaque in a non-culprit artery were studied in 581 patients with stable angina pectoris (SAP) or acute coronary syndrome (ACS), who were admitted for coronary angiography. By using virtual-histology intravascular ultrasound (VH-IVUS) the extensiveness, composition and vulnerability of coronary plaques were assessed of either a segment or lesion. The extent / degree of coronary atherosclerosis was determined by measuring plaque burden, plaque volume, minimal luminal area in segments and the occurrence of lesions with ≥70% plaque burden or ≤4.0mm^2 minimal lumen area. The composition / phenotype of the atherosclerotic plaque was assessed by identifying plaque components as dense calcium, necrotic core, fibro-fatty and fibrous tissue. The vulnerability was characterized by the presence of VH-IVUS derived thin-cap fibroatheroma (TCFA) lesions. Additionally, patients' clinical information was collected and pre-procedural blood samples were drawn for biomarker determination. By collecting these data from various sources we aim to improve our understanding on the role of external influences and circulating biomarkers in the development of (vulnerable) coronary atherosclerotic plaques.

Furthermore, clinical follow-up of these patients from inclusion until 1 year was performed to explore the prognostic value of (novel) circulating biomarkers in predicting recurrent adverse events.

In **Chapter 2** the relationship between cigarette smoking and coronary atherosclerosis is described. With regard to degree of atherosclerosis, current smokers had slightly higher coronary plaque burden than never smokers, especially in patients presenting with ACS. With respect to plaque composition, fibro-fatty tissue was the prevailing compound in the coronary arteries of cigarette smokers. Lastly, cigarette smoking was not associated with coronary plaque vulnerability. Lack of such an association may in part be explained by the possibility that rather plaque erosion than vulnerable plaque rupture is the underlying mechanism for coronary events in smoking CAD patients, as suggested by earlier, histopathological studies. Although sufficient evidence is available demonstrating that smoking directly impacts multiple aspects in atherosclerosis, less is currently known about the associations of smoking and the in-vivo plaque structure within the coronary artery itself. Altogether, these findings modestly contribute towards a better understanding of the complex pathophysiologic relationship between cigarette smoke exposure and CAD.

Chapter 3 highlights fibrinogen as a biomarker of interest in coronary atherosclerosis to provide additional insight into the nature of relationship with CAD. The association of plasma fibrinogen with extent, composition and vulnerability of coronary atherosclerosis, as well as with 1-year cardiovascular outcome, was investigated. Higher circulating fibrinogen levels were associated - not independently of C-reactive protein (CRP) - with higher degree of coronary atherosclerotic plaque burden, particularly in patients presenting with ACS. Substantial associations of fibrinogen with coronary plaque composition, vulnerability and 1-year cardiovascular outcome were absent. Overall, these results point towards involvement of fibrinogen in progression of coronary atherosclerotic plaque formation, especially in ACS patients, rather than in plaque vulnerability or clinical events directly.

In **Chapter 4** the association between adiponectin and VH-IVUS coronary plaque characteristics, as well as 1-year cardiovascular outcome, was studied to further elucidate the pathophysiology of adiponectin in patients with established CAD. Adiponectin levels were associated with 1-year mortality, mainly in patients presenting with SAP. In SAP patients, adiponectin was associated with features of plaque vulnerability. The exact mechanism behind the positive association between adiponectin and coronary plaque (in)stability warrants further investigation specifically in SAP patients.

Chapter 5 evaluates four circulating chemokines in relation to coronary atherosclerosis, as well as 1-year clinical outcome. Circulating regulated upon activation normal T-cell expressed and secreted (RANTES) was demonstrated to be a promising biomarker, that correlated with coronary plaque burden, vulnerability and cardiovascular outcome (in particular death and re-occurrence of ACS).

Chapter 6 describes the relationship between eight different circulating cytokines and coronary plaque characteristics assessed with VH-IVUS. Higher circulating tumor necrosis factor alpha (TNF-α, pro-inflammatory cytokine) was associated with higher coronary plaque burden and with plaque vulnerability in SAP patients; and lower circulating interleukin-10 (IL-10, anti-inflammatory cytokine) in SAP and ACS patients. Furthermore, the relationship between the investigated cytokines and 1-year cardiovascular outcome has been investigated as well. However, none showed prognostic value for clinical outcome. Taken together, these results imply that the potential effect of TNF-α and IL-10 on coronary atherosclerosis cannot automatically be extrapolated to clinical outcome.

Chapter 7 focuses on two renal biomarkers in relation to coronary atherosclerosis and occurrence of 1-year recurrent clinical events. In patients with a normal renal function, plasma cystatin C (a glomerular marker) and plasma neutrophil gelatinase-associated lipocalin (a tubular marker) showed an inverse relationship with high-risk coronary lesions such as TCFA plaques and lesions with large plaque burden (>70%). However, in patients with an impaired renal function, these 'protective' effects were absent, leading to novel (pathophysiological) insights. Furthermore, only higher cystatin C levels in exclusively patients with renal dysfunction predicted 1-year adverse clinical events. In other words, cystatin C appears to carry most potential of the two renal biomarkers with regard to cardiovascular risk prediction, but only in patients with deteriorated kidney function.

In conclusion, the study of circulating biomarkers in relation to VH-IVUS derived coronary plaque characteristics resulted in distinct findings, which led to new pathophysiological insights and hypotheses. Also, the exploration of the biomarkers' capability in predicting recurrent events showed that adiponectin, RANTES and cystatin C are promising in the road towards improving secondary cardiovascular risk prediction. Lastly, we have learned the importance of stratifying patients on treatment indication (ACS versus SAP) within the spectrum of CAD to account for their different pathophysiological entities.

When investigating the association between the aforementioned biomarkers and coronary plaque characteristics, it must be acknowledged that the *ATHEROREMO-IVUS*

study design does not permit causal inference. In this cross-sectional design both coronary plaque characteristics were measured and blood samples were drawn simultaneously. The possibility that the biomarkers may be the result of clinical presentation rather than its cause, cannot be excluded. Nevertheless, this study provides novel data on biomarkers in relation to in-vivo assessment of the coronary arterial wall (i.e. more accurate measures of coronary atherosclerosis) and have herewith resulted in new hypotheses complementary to existing studies investigating these associations. Thus, although causal inference is debatable, the generated data by this unique study may still be informative, since they provide additional insights into the complex pathophysiological relation between biomarkers and CAD.

Furthermore, in general it can be stated that the association between the biomarkers and 1-year cardiovascular outcome was not substantial. This may have, in part, been caused by a limited number of clinical events, leading to a lack of statistical power to demonstrate strong associations. Another possible explanation may include the fact that the magnitude of effect of the investigated biomarkers separately is small in the context of this multifactorial disease.

2. Towards enhanced risk stratification in established CAD by studying blood biomarker patterns in detail.

In the second part of this thesis, the behavioral temporal pattern after an acute coronary syndrome admission of novel and established cardiovascular biomarkers is described during 1-year follow-up. This has been performed within the *BIOMarker study to identify Acute risk of a Coronary Syndrome (BIOMArCS)* consisting of 844 patients, of whom frequently repeated blood sample measurements were drawn shortly after ACS admission until 1 year. Utilizing a high-frequency blood sampling design enables a unique insight in the value of these biomarkers for purposes of long-term secondary cardiovascular risk prediction.

Chapter 8 concentrates on growth differentiation factor-15 (GDF-15) patterns and individual variability in 111 ACS patients. Differences in temporal changes between patients with (cases) and without (controls) a recurrent event (composite of cardiovascular mortality, myocardial infarction or unstable angina pectoris requiring urgent coronary revascularization) during follow-up were of particular interest. It appeared that post ACS patients experiencing a recurrent event had systematically higher GDF-15 levels during 1-year follow-up than their event-free counterparts with otherwise similar clinical characteristics. Additionally, GDF-15 showed low within-individual variability. Altogether, postdischarge blood sampling of GDF-15 might be used throughout the course of 1 year to improve prognostication, whereas, in

view of the low within-individual variation, the number of repeated measurements might be limited.

In **Chapter 9** the temporal evolution of established cardiovascular biomarkers (high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides, total cholesterol, cardiac Troponin T (TnT), NT-proBNP and CRP) in diabetic post ACS patients is studied with their respect to inter-ethnic differences. Thus far, most cardiovascular biomarkers have been merely validated in Caucasians, and little is known about their generalizability to other ethnic groups. Especially in Asia, where CAD now is an upcoming epidemic, the need for biomarker validation is warranted. Therefore, the 1-year trajectories following the index ACS event were displayed for each biomarker and compared between 48 Caucasian (Dutch) and 48 clinically matched Chinese patients. The observational findings revealed that Caucasian patients persistently possessed higher levels of LDL, total cholesterol, triglycerides, TnT and CRP than Chinese patients. No differences in HDL and NT-proBNP were observed between the two cohorts. This exploratory analysis could give more insight into blood biomarker related differences among ethnic groups and underscores the fact that it may not be convenient to solely apply findings from most Western cohorts to Chinese individuals.

Chapter 10 examines the short- (<30 days) and long-term (30 days – 1 year) time course of protein blood biomarkers that stimulate the intracellular mitogen-activated protein kinase (MAPK) cascade in 135 post ACS patients. Temporal differences between patients with (cases) and without (controls) a recurrent event (composite of cardiovascular mortality, myocardial infarction or unstable angina pectoris requiring urgent coronary revascularization) were particularly investigated. Serum levels of angiopoietin-1 (ANG-1), protease-activated receptor-1 (PAR-1) and bone morphogenetic protein-6 (BMP-6) were significantly higher in patients developing a recurrent event than their matching controls, but only in the first 30 days following ACS. Hence, prognostic abilities of these signaling proteins - that may serve as novel biomarkers in the field of CAD - should be further evaluated in the acute phase after ACS.

Concluding, the exploration of novel blood biomarkers reflecting relevant pathophysiological processes in CAD may lead to identification of markers that might be useful for risk prediction and even be of interest as therapeutic targets. In this respect, our investigation led to promising findings of GDF-15, ANG-1, PAR-1 and BMP-6 as the result of studying their detailed patterns over time. Also, relevant and sustainable differences have been discovered between Caucasian and Chinese patients for established cardiovascular

biomarkers, which emphasizes the need for an ethnicity-specific approach.

Temporal biomarker patterns have been compared between cases and controls according to a case-cohort design within *BIOMArCS*. For reasons of efficiency, biomarkers were determined in all cases who reached the study endpoint, but in a limited number of event-free patients. This analysis approach allows to reveal clinically relevant differences with sufficient statistical power in an efficient way. The strength of the *BIOMArCS* study lies in the fact that serial measurements have been obtained directly after an ACS (index event) and before an ACS (recurrent event), which enables accurate observations of alterations in biomarker patterns prior to an event. This is in contrast to previous studies with single measurements of blood biomarkers, often obtained in the early, acute phase of an acute ischemic event. Moreover, the collection of multiple measurements represents a more stable state during a longer period, less affected by accidental factors than single measurements.

It is important to emphasize the observational character of *BIOMArCS* and its substudies, implying that causal inference cannot be demonstrated. Whether the investigated blood biomarkers merely reflect pathways of disease in CAD, or directly contribute to coronary pathophysiological cascades as mediators, remains to be unravelled. Also, it must be noted that genetic, environmental and specific clinical information of the study participants, possibly influencing circulating blood biomarkers, such as diet or infarct size, was lacking. These possible confounders were - as far as possible - addressed by matching patients on clinical characteristics and admission diagnosis. Lastly, by specifically studying post ACS patients, it is not known whether these findings can be extrapolated to other CAD patients or applied to the general population in primary prevention.

3. Towards enhanced risk stratification and improved treatment benefit in established CAD by genetic polymorphisms.

In the third part of this thesis, the relationship between genetic polymorphisms and cardiovascular outcome has been studied in order to investigate its usefulness in cardiovascular risk stratification. Furthermore, the value of some genetic variants for targeting therapy in patients who would benefit most from ACE-inhibitors has been assessed as well. These associations have been investigated in the *PERindopril GENEtic Associations (PERGENE) study*, a substudy of the *EURopean trial On reduction of cardiac events with Perindopril in stable coronary Artery disease (EUROPA)*. The *EUROPA* trial was a randomized, double-blind, placebo-controlled clinical trial designed to assess the effect of the ACE-inhibitor

perindopril on adverse cardiovascular outcome in 12218 patients with stable CAD. *PERGENE* was a cardiovascular pharmacogenetic substudy investigating polymorphisms that might modify the treatment benefit of the ACE-inhibitor perindopril on adverse cardiovascular outcome. The latter chapter (chapter 13) in this part describes the association between genetic polymorphisms and coronary plaque characteristics, as part of the previously mentioned *ATHEROREMO-IVUS* study.

Chapter 11 encompasses a comprehensive analysis of seven functional variants in the vascular endothelial growth factor (VEGF) and kinase insert domain-containing receptor (KDR) genes in relation to cardiovascular outcome as well as treatment benefit entailed by the ACE-inhibitor perindopril. In a total of 8711 investigated stable CAD patients, no associations were found between VEGF/KDR polymorphisms or their haplotypes with 4-year cardiovascular outcome. On the other hand, multiple VEGF/KDR polymorphisms, especially when combined into one pharmacogenetic score, modified the treatment effect of perindopril on adverse cardiovascular outcome. Altogether, these findings do not support that VEGF/KDR variants are useful for risk assessment in stable CAD, but might rather be used to increase treatment benefit of the ACE-inhibitor perindopril with regard to prevention of adverse cardiovascular outcome. A possible interaction between VEGF/KDR variants and ACE-inhibitor therapy has never been investigated before in clinical studies and therefore revealed novel information on this matter.

Chapter 12 focuses on two α-adducin (ADD1) polymorphisms (rs4961 and rs4962) in relation to cardiovascular outcome, treatment effect by the ACE-inhibitor perindopril and hypertension. In a total of 9327 patients with stable CAD, ADD1 polymorphisms were not associated with 4-year cardiovascular outcome. Nor did they modify treatment benefit of perindopril in terms of blood pressure reduction or cardiovascular risk reduction. With regard to hypertension, only minor allele carriers of the rs4963 polymorphism had a significanly lower prevalence of hypertension than their wild type homozygote counterparts. It can be stated – based on these findings – that ADD1 polymorphisms do not provide a window of opportunity for possible future cardiovascular risk prediction, nor for individualized tailoring of ACE-inhibitor therapy in patients with stable CAD.

In **Chapter 13** a common haptoglobin (Hp) polymorphism has been studied by assessing its protein phenotypes (Hp 1-1, Hp 2-1, Hp 2-2) in a total of 585 patients undergoing coronary angiography for SAP or ACS. The degree and composition of coronary atherosclerosis in these patients has been determined by IVUS-VH and near-infrared spectroscopy (NIRS), enabling

to investigate the relationship between Hp phenotype and invasively-measured coronary plaque characteristics. There was a positive association between Hp 2 phenotypes (Hp 2-1 and Hp 2-2) and the degree of atherosclerosis in ACS patients, meaning that ACS patients possessing a Hp 2 phenotype are at higher risk for more advanced coronary atherosclerosis than ACS patients with a Hp 1 phenotype. Thus, genetic differences in the endogenous anti-oxidant status, as reflected by the Hp phenotype, may be of importance in risk stratification of patients suffering from CAD.

Overall, by examining specific genetic variants within the range of CAD, two main objectives have been addressed in this part of the thesis. First, the specified polymorphisms were valued on their potential capability in cardiovascular risk prediction. While VEGF/KDR and ADD1 polymorphisms did not seem promising in this field, haptoglobin polymorphisms may have shown to be useful in coronary risk stratification. Second, the role of the investigated polymorphisms in optimizing treatment benefit of ACE-inhibitors in stable CAD patients was evaluated. In this respect, an interaction was found between treatment effect of the ACE-inhibitor perindopril on cardiovascular outcome and VEGF/KDR polymorphisms, but not ADD1 polymorphisms. This knowledge might be useful information towards the road of targeting pharmacological treatment at an individual level.

It must be noted that the VEGF/KDR and ADD1 polymorphisms have been analyzed within a well-designed placebo-controlled double-blinded randomized controlled trial with a large sample size and relatively long follow-up. However, some aspects warrant consideration. The lack of association between the separately analyzed polymorphisms and 4-year cardiovascular outcome might be explained by the fact that it is likely that the contribution of a single single-nucleotide polymorphism (SNP) to prognosis is minimal; an individual SNP might not be able to carry prognostic information alone. Therefore, specific combinations of several SNPs were reconstructed into haplotypes in order to gain more power. Still, there was no significant association present between any of the haplotypes and cardiovascular outcome. It must also be mentioned that the generalizability of the results has limitations regarding the type of patients and type of agent, meaning that these findings may not be directly extrapolated to all CAD patients and all ACE-inhibitors. The *PERGENE* study population consisted of predominantly male Caucasian patients with stable CAD, receiving perindopril. Therefore, these results must be carefully interpreted with regard to women, other ethnicities, other CAD types and different ACE-inhibitors.

15 *Main conclusions*

15 Main conclusions

A couple of lessons can be learned from this thesis and may be summarized as follows:

PART 1:

- Recurrent adverse cardiovascular events still commonly occur in patients with established coronary artery disease, demanding an ongoing gain in knowledge, improved risk stratification / prediction tools and enhanced treatment strategies.
- By linking external influences and circulating blood biomarkers to invasively measured coronary plaque characteristics, additional pathophysiological insights may be provided.
- Cigarette smoking and circulating fibrinogen rather contribute to the degree of coronary atherosclerotic burden than coronary plaque vulnerability, especially in ACS patients.
- Conversely, circulating adiponectin has involvement in coronary plaque vulnerability specifically in SAP patients.
- Several chemokines (MCP-1, MIP-1α, RANTES) and cytokines (TNF-α, IL-10) are associated with various aspects of degree, composition and vulnerability of coronary atherosclerosis.
- Higher circulating levels of renal biomarkers (cystatin C and NGAL) are related to less high-risk coronary lesions, but not in CAD patients with an impaired renal function.
- Adiponectin, RANTES and cystatin C carry predictive value for 1-year adverse clinical events in established CAD. Thus, these circulating biomarkers are potential candidates whose incremental value should be further evaluated in prediction models of recurrent events in CAD patients.
- Stratifying patients on CAD type (ACS versus SAP) is essential to account for their different pathophysiological way of acting.

PART 2:

- Exploring novel blood biomarkers reflecting relevant pathophysiological processes in CAD may lead to identification of markers that might be useful for risk prediction and even be of interest as therapeutic targets.
- Previous blood biomarker studies within the field of CAD mainly focus on single measurements in the early, acute phase of an acute ischemic event.

- Learning about the temporal evolution of blood biomarkers after ACS by means of high-frequency blood sampling can be useful in 4 ways:
  - Observing the behavior and stability of the marker in the acute phase and on the long term after an event.
  - Observing its behavioral pattern before a recurrent event.
  - Comparing biomarker patterns between patients with and without a recurrent event.
  - Determining within- and between-patient variability of the marker.
- Post ACS patients experiencing a recurrent event have stable and systematically higher GDF-15 levels during 1-year follow-up than their matching controls. Therefore, blood sampling of GDF-15 might be used throughout the course of 1 year post ACS to improve prognostication with a limited number of necessary repeated measurements.
- On the other hand, blood biomarkers stimulating intracellular mitogen-activated protein kinase (ANG-1, PAR-1, BMP-6) only have predictive value in the acute phase for post ACS patients experiencing a recurrent event.
- Relevant and sustainable differences exist in long-term temporal patterns of established cardiovascular biomarkers (LDL, total cholesterol, triglycerides, troponin T and CRP) between Caucasian and Chinese post ACS patients, emphasizing the need for an ethnicity-specific approach.

PART 3:

- Studying the relationship between genetic polymorphisms and cardiovascular outcome is useful to determine the utility of genetics in secondary cardiovascular risk stratification.
- Variants in the vascular endothelial growth factor (VEGF), kinase insert domain-containing receptor (KDR) and α-adducin (ADD1) genes do not carry prognostic information on 4-year adverse cardiovascular outcome in stable CAD patients.
- Genetic differences in the endogenous anti-oxidant status, as reflected by the haptoglobin phenotype, may be of importance in determining a higher risk for more advanced coronary atherosclerosis in ACS patients.
- Genetic variants can be utilized to target pharmacological treatment in those who would benefit most and to herewith contribute to personalized medicine.
- Multiple VEGF/KDR polymorphisms modified the treatment effect of perindopril on adverse cardiovascular outcome in patients with stable CAD.

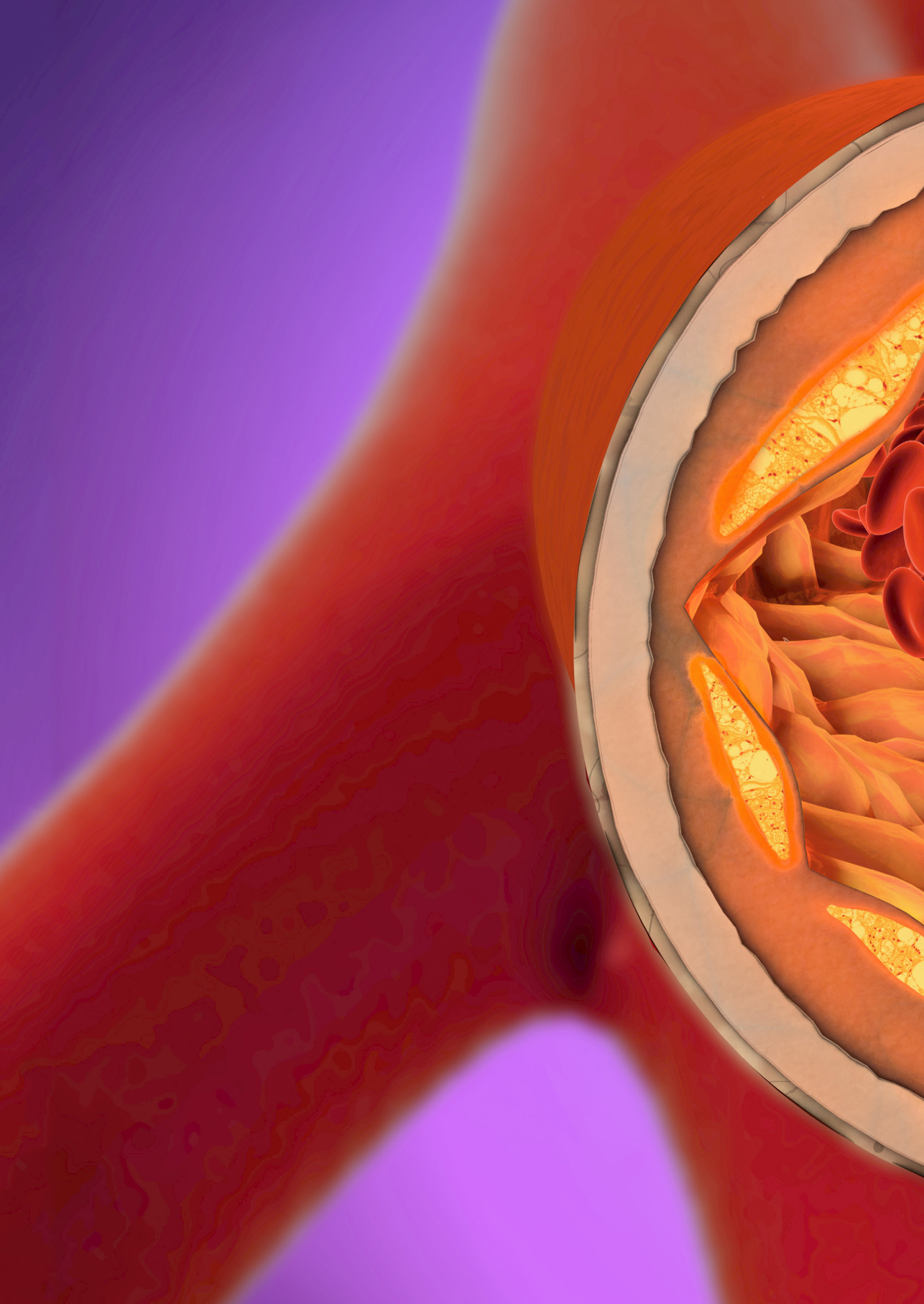
16 *Clinical perspectives and future directions*

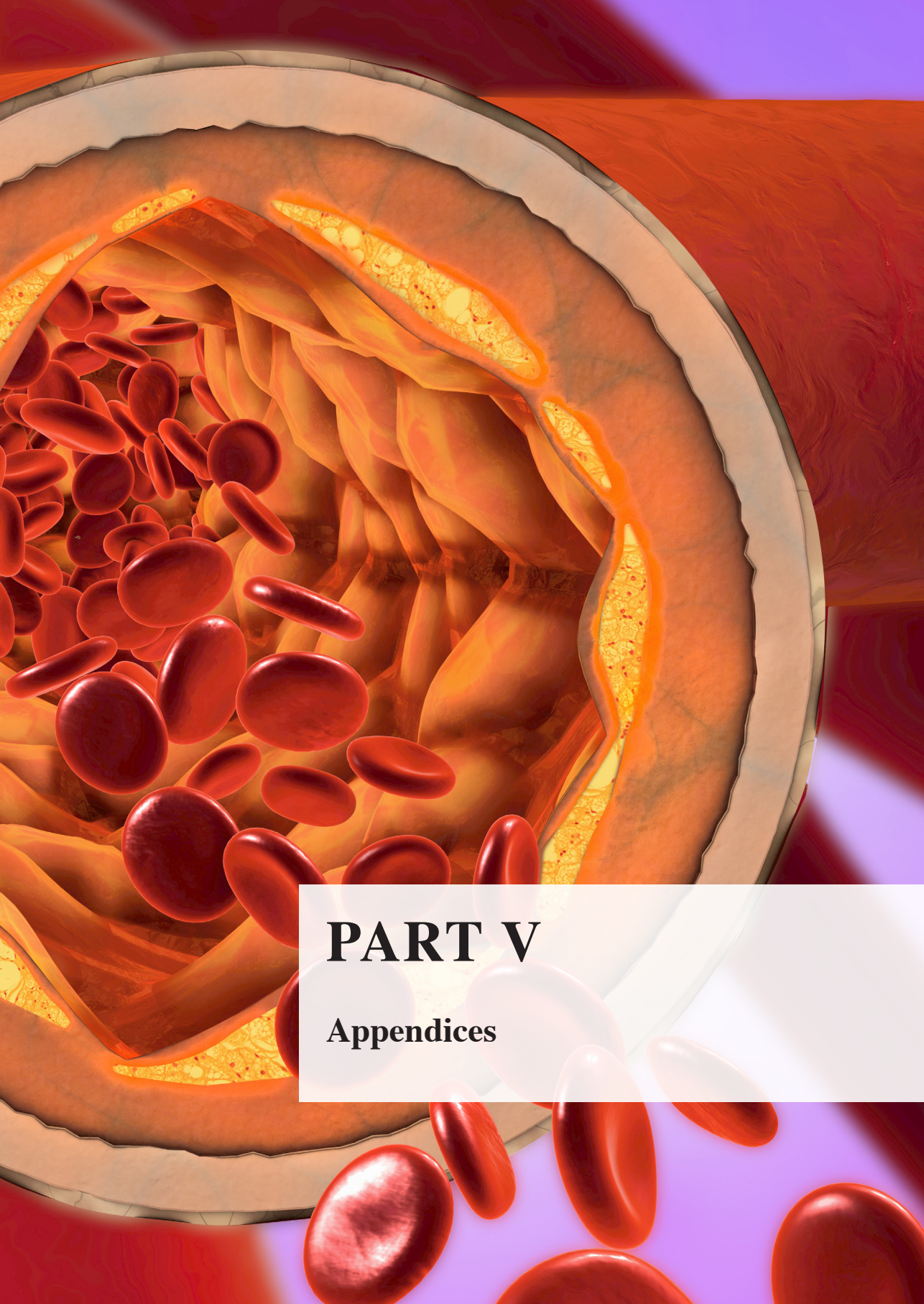
16 Clinical perspectives and future directions

Ongoing research in coronary pathophysiology over the past decennia has led to crucial discoveries, responsible for a 'paradigm shift' in the conceptualization of underlying mechanisms in CAD. The investigation of blood biomarkers has been essential in this respect. Our current view on CAD has shifted from a chronic, steady and progressive metabolic process towards a more multifactorial, dynamic disease. Further improvement in our understanding of coronary pathophysiology could be established by continuing to investigate the value of established biomarkers and persist in searching for novel biomarkers. Eventually, this ongoing conceptual development could lead to new potential targets for therapeutic interventions.

Bearing this theory in mind, blood biomarkers could also contribute towards a disease management transition, in which CAD patients are no longer considered to be a homogeneous group requiring similar long-term pharmacotherapeutic treatment. Instead, CAD patients should be regarded as a heterogeneous group with a more individual approach, considering their different composition of constantly dynamic underlying pathophysiological components. In this respect, blood biomarkers could potentially fulfill a key role, since they possess the ability to quite specifically reflect the pathophysiological alterations through varying circulating levels over time. Ultimately and ideally, the assessment of blood biomarkers in clinical practice would be an easy and fast application tool to non-invasively gain information on the individual coronary risk of the patient. Hereby it could serve for risk stratification to identify 'high risk' patients in order to take early preventive measures before the actual occurrence of a recurrent event. Also, genetic variants could be a valuable addition to individually decide who benefits most from certain pharmacotherapeutic interventions. With current available tools for risk stratification, it is not possible to predict recurrent events in a specific and short future timeframe or to guide therapy towards good responders. It must be acknowledged that the observational, exploratory and hypothesis-generating findings of this thesis do not aim a direct implementation in clinical practice. They rather provide new pathophysiological insights and lessons to be learned for setting up future studies, that aim for directly applicable results in medical practice.

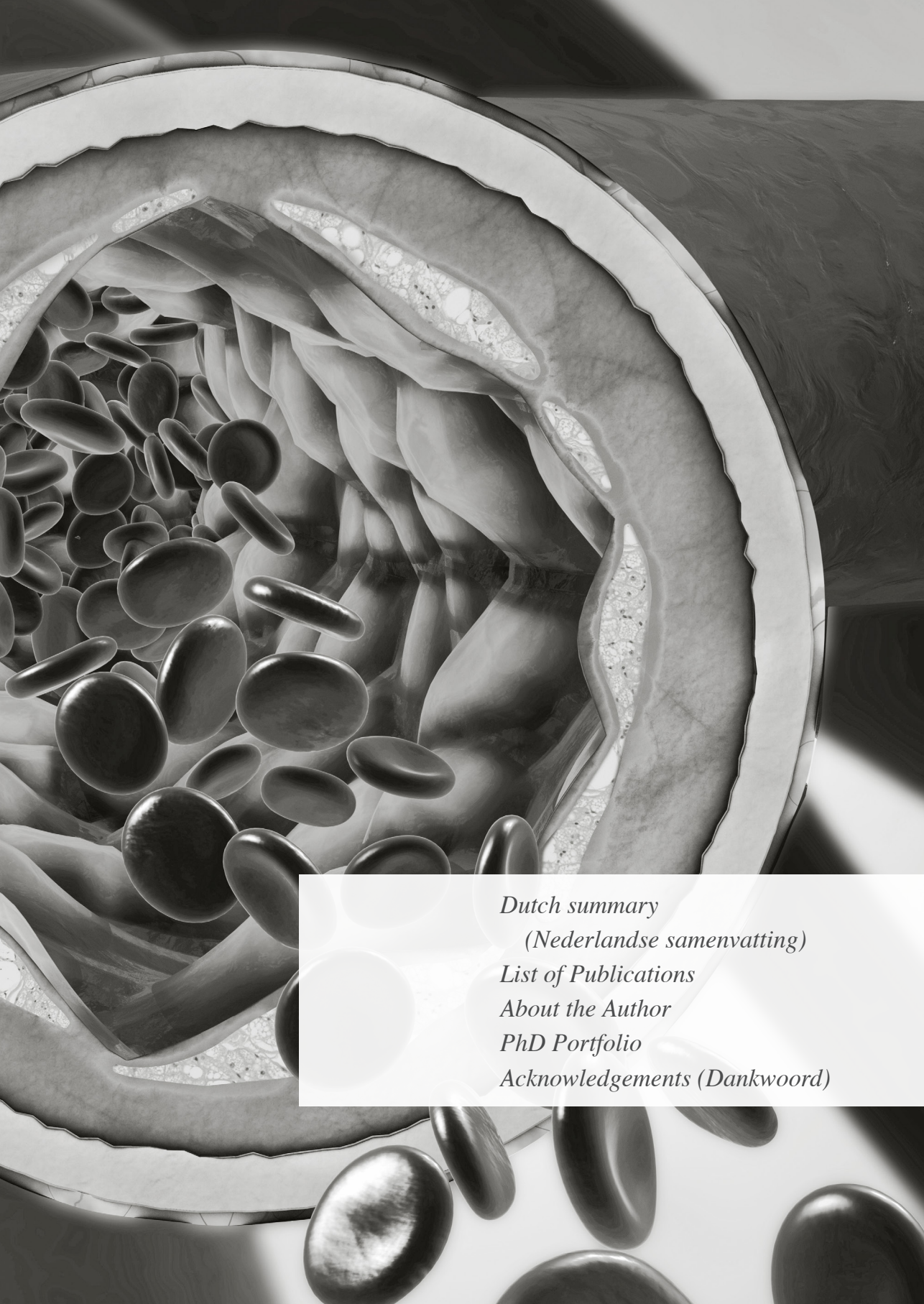
Concluding, an optimal risk stratification score for daily practice in established CAD would ideally be an individual approach and consist of clinical characteristics combined with intracoronary imaging, genetics and (repeated) measurements of multiple biomarkers carrying the most prognostic information. By identifying 'high risk' patients who would most likely benefit from (targeted) therapy in advance, adverse cardiac event reduction may be established. The exploratory findings of this thesis need to be combined in large, prospective clinical studies and further explored on their potential additional value in cardiovascular risk prediction over established clinical covariates. Thus, much progress remains to be made on this field in the future era of 'personalized medicine'.





PART V

Appendices



Dutch summary
List of publications
About the Author
PhD Portfolio
Acknowledgements

Dutch summary (Nederlandse samenvatting)

Herhaaldelijke ongunstige cardiovasculaire events komen nog altijd voor in een aanzienlijk deel van patiënten met coronaire hartziekte, ondanks verbeterde technologieën en een toegenomen aantal behandelopties. De behoefte aan een betere risicovoorspelling bij patiënten met coronairlijden is daarom de grootste 'drive' geweest voor dit proefschrift. Door onze kennis te vergroten op het gebied van bloedbiomarkers en genetische polymorfismen, die een rol lijken te spelen in de pathofysiologie van coronairlijden, wordt gestreefd naar een optimale risicostratificatie in patiënten met verworven coronaire hartziekte. In dit hoofdstuk worden de hoofdresultaten van dit proefschrift samengevat en in een bredere context geplaatst.

1. Een beter begrip over coronair atherosclerose in mensen creëren door bloedbiomarkers te linken aan intracoronaire beeldvorming.

In het eerste deel van dit proefschrift worden biologische processen - met een mogelijke betrokkenheid bij atherosclerose - bestudeerd in relatie tot coronair atherosclerose door middel van intracoronaire beeldvorming binnen de *European Collaborative Project on Inflammation and Vascular Wall Remodeling in Atherosclerosis – Intravascular Ultrasound (ATHEROREMO-IVUS) study*. De karakteristieken van elke atherosclerotische plaque in een 'non-culprit' (niet ziek) coronairvat zijn in 581 patiënten bestudeerd, die verwezen waren voor coronairangiografie omwille van stabiele angina pectoris (SAP) of acuut coronairsyndroom (ACS). Door gebruik te maken van 'virtual-histology intravascular ultrasound' (VH-IVUS) werd de uitgebreidheid, compositie (samenstelling) en kwetsbaarheid van de plaques bepaald in een segment of lesie van een coronairvat. De uitgebreidheid / omvang van coronair

atherosclerose werd vastgesteld door het meten van 'plaque burden', 'plaque volume', 'minimal lumen area' in segmenten en de aanwezigheid van het aantal lesies met ≥70% 'plaque burden' of ≤4.0mm^2 'minimal lumen area'. De compositie (het fenotype) van de atherosclerotische plaque werd vastgesteld door de bepaling van verschillende plaque componenten zoals 'dense calcium', 'necrotic core', 'fibro-fatty' en 'fibrous' weefsel. De 'vulnerability' (kwetsbaarheid) van een plaque werd gekarakteriseerd door de aanwezigheid van VH-IVUS bepaalde 'thin-cap fibroatheroma' (TCFA) lesies. Daarnaast werd klinische informatie over de patiënten verzameld en werden voor de procedure bloedbuizen afgenomen ten behoeve van biomarkerbepalingen. Het doel van het verzamelen van al deze informatie is om een beter begrip te creëren over de rol van externe invloeden en circulerende bloedbiomarkers in de ontwikkeling van (kwetsbare) coronair atherosclerotische plaques. Bovendien werd een klinische follow-up van de patiënten uitgevoerd vanaf inclusie tot 1 jaar om de prognostische waarde van (nieuwe) circulerende bloedbiomarkers te onderzoeken in het voorspellen van herhaaldelijke ongunstige events.

In **Hoofdstuk 2** wordt de relatie tussen roken en coronair atherosclerose beschreven. Met betrekking tot de omvang van de atherosclerose, bleken huidige rokers iets meer coronair 'plaque burden' te hebben dan nooit-rokers, vooral in patiënten die zich presenteerden met ACS. Ten aanzien van de plaque compositie, bleek 'fibro-fatty' weefsel het overheersende type bestanddeel te zijn in de coronairen van huidige rokers. Tot slot werd geen associatie gevonden tussen roken en 'vulnerability' van de coronaire plaque. Het ontbreken van een dergelijke associatie zou deels verklaard kunnen worden door de mogelijkheid dat plaque erosie - en niet zozeer plaqueruptuur - het onderliggende mechanisme zou kunnen zijn voor coronaire events in rokende patiënten met coronairlijden, zoals al eerder werd gesuggereerd door histopathologische studies. Alhoewel voldoende bewijs voorhanden is dat roken op allerlei vlakken invloed uitoefent op het proces van atherosclerose, is er momenteel minder bekend over associaties tussen roken en de 'in vivo' plaquestructuur binnen het coronairvat zelf. Alles tezamen leveren deze bevindingen een geringe bijdrage aan een beter begrip over de complexe pathofysiologische verhouding tussen roken en coronairlijden.

Hoofdstuk 3 accentueert fibrinogeen als mogelijke interessante bloedbiomarker in coronair atherosclerose om aanvullend inzicht te creëren in de relatie tot coronairlijden. De associatie tussen fibrinogeen met uitgebreidheid, compositie en kwetsbaarheid van coronair atherosclerose, alsmede met cardiovasculaire uitkomsten binnen 1 jaar, werd onderzocht. Hieruit bleek dat hoe hoger de circulerende fibrinogeenwaarden waren, hoe

groter de uitgebreidheid van coronair atherosclerose was, vooral bij patiënten met ACS. Deze relatie was echter niet onafhankelijk van 'C-reactive protein' (CRP). Er werden geen beduidende associaties gevonden tussen fibrinogeen en coronaire plaque compositie, plaque 'vulnerability' of cardiovasculaire uitkomsten binnen 1 jaar. Al met al duiden deze resultaten erop dat fibrinogeen eerder een rol speelt in coronaire atherosclerotische plaque progressie, met name in ACS patiënten, dan in de 'vulnerability' van de plaque of een directe invloed heeft op coronaire events.

In **Hoofdstuk 4** is de associatie bestudeerd tussen adiponectine met zowel VH-IVUS afgeleide coronaire plaque karakteristieken als 1-jaar cardiovasculaire uitkomsten, om de pathofysiologische rol van adiponectine in patiënten met coronaire hartziekte verder te verduidelijken. Adiponectine levels bleken geassocieerd te zijn met 1-jaars mortaliteit in alle patiënten met coronairlijden, maar vooral gedreven door patiënten die zich presenteerden met SAP. Ook bleek alleen in SAP patiënten adiponectine geassocieerd te zijn met plaque karakteristieken omtrent 'vulnerability'. Het exacte onderliggende mechanisme wat betreft de associatie tussen adiponectine en coronaire plaque (in)stabiliteit dient nader te worden onderzocht in met name SAP patiënten.

Hoofdstuk 5 beschrijft vier circulerende chemokines in relatie tot coronair atherosclerose en klinische uitkomsten binnen 1 jaar. Circulerende 'regulated upon activation normal T-cell expressed and secreted' (RANTES) kwam uit de resultaten als een veelbelovende biomarker, welke correleerde met coronaire 'plaque burden', 'plaque vulnerability' en cardiovasculaire uitkomsten (met name mortaliteit en herhaaldelijk voorkomen van ACS).

Hoofdstuk 6 beschrijft de relatie tussen acht verschillende circulerende cytokines and coronaire plaque karakteristieken, zoals bepaald met VH-IVUS. Hogere circulerende waarden van 'tumor necrosis factor alpha' (TNF-α, pro-inflammatoire cytokine) bleken geassocieerd te zijn met meer coronaire 'plaque burden' en 'plaque vulnerability' in alleen SAP patiënten; en lagere circulerende waarden van interleukine-10 (IL-10, anti-inflammatoire cytokine) in alle patiënten met coronairlijden. Daarnaast is de relatie van de onderzochte cytokines bekeken met 1-jaar cardiovasculaire uitkomsten. Echter, geen van allen toonde enige prognostische waarde voor klinische uitkomst. Alles bij elkaar genomen, wijzen deze resultaten erop dat een potentieel effect van TNF-α en IL-10 op coronair atherosclerose niet direct geëxtrapoleerd kan worden naar klinische uitkomst.

Hoofdstuk 7 focust op twee renale biomarkers in relatie tot coronair atherosclerose en voorkomen van herhaaldelijke ongunstige klinische events binnen 1 jaar. In patiënten

met een normale nierfunctie, bleken plasma 'cystatine C' (een glomerulaire marker) en plasma 'neutrophil gelatinase-associated lipocalin' (een tubulaire marker) een omgekeerde relatie te hebben met hoog-risico coronaire laesies zoals 'TCFA' plaques en laesies met veel 'plaque burden' (>70%). Echter, bij patiënten met een verslechterde nierfunctie, konden deze 'beschermende' effecten niet aangetoond worden, wat heeft geleid tot nieuwe (pathofysiologische) inzichten. Bovendien bleek alleen bij patiënten met een slechte nierfunctie hogere cystatine C levels van voorspellende waarde te zijn voor een ongunstige klinische uitkomst binnen 1 jaar. Met andere woorden, cystatine C blijkt van de twee renale biomarkers de meeste potentie te hebben met betrekking tot cardiovasculaire risicopredictie, maar alleen in patiënten met een verslechterde nierfunctie.

Concluderend heeft het bestuderen van circulerende biomarkers in relatie tot VH-IVUS afgeleide coronaire plaque karakteristieken geresulteerd in verschillende bevindingen, die tot nieuwe pathofysiologische inzichten en hypothesen heeft geleid. Tevens heeft het onderzoek naar het vermogen van de biomarkers in het voorspellen van herhaaldelijke events laten zien dat adiponectine, RANTES en cystatine C veelbelovend zijn in de weg naar het verbeteren van secundaire cardiovasculaire risicopredictie. Tot slot hebben we van onze resultaten geleerd dat het van belang is om patiënten op behandelindicatie (ACS versus SAP) te stratificeren binnen het spectrum van coronaire hartziekten om rekening te houden met diens verschillende pathofysiologische entiteiten.

In het onderzoek naar de associatie tussen de eerder genoemde biomarkers en coronaire plaque karakteristieken, moet erkend worden dat de opzet van de *ATHEROREMO-IVUS* studie niet in staat stelt om causale inferentie aan te tonen. In deze cross-sectionele studieopzet zijn tegelijkertijd coronaire plaque karakteristieken middels VH-IVUS bepaald en bloedbuizen afgenomen. De mogelijkheid dat de biomarkers eerder het gevolg zijn van de klinische presentatie dan de oorzaak, kan daarom niet worden uitgesloten. Desalniettemin levert dit onderzoek nieuwe data aan over biomarkers in relatie tot een in-vivo beoordeling van de coronaire arteriële wand (oftewel een nauwkeurigere meting van coronair atherosclerose) en heeft daarbij geresulteerd in nieuwe hypotheses aanvullend op reeds bestaande studies, die deze associaties op andere wijzen hebben onderzocht. Alhoewel causale inferentie betwist kan worden, zijn de tot stand gekomen data uit dit onderzoek toch uniek en informatief, aangezien ze nieuwe inzichten hebben verschaft in de complexe pathofysiologische verbanden tussen biomarkers en coronaire hartziekten.

Daarnaast kan over het algemeen gesteld worden dat de associatie tussen de biomarkers

en cardiovasculaire uitkomsten binnen 1 jaar niet substantieel was. Dit kan deels te maken hebben gehad met een beperkt aantal klinische events in deze studie, wat heeft geleid tot een gebrek aan statistische power om sterke associaties aan te kunnen tonen. Een andere mogelijke verklaring is het feit dat het aandeel van iedere biomarker afzonderlijk relatief klein is in de context van een multifactoriële aandoening.

2. Een betere risicostratificatie bij verworven coronaire hartziekten door patronen in bloedbiomarkers te bestuderen.

In het tweede deel van dit proefschrift wordt het gedragspatroon beschreven van nieuwe en reeds bestaande cardiovasculaire biomarkers gedurende 1 jaar na het doormaken van een ACS event. Dit is uitgevoerd binnen de *BIOMarker study to identify Acute risk of a Coronary Syndrome (BIOMArCS)*, bestaande uit 844 patiënten, in wie frequente herhaaldelijke bloedafnames hebben plaatsgevonden direct na een ACS tot aan 1 jaar. Door gebruik te maken van een schema met hoog-frequente bloedafnames kan op een unieke wijze inzicht worden verkregen in de waarde van de onderzochte biomarkers voor secundaire cardiovasculaire risicopredictie op de langere termijn.

Hoofdstuk 8 concentreert zich op growth differentiation factor-15 (GDF-15) patronen en diens individuele variabiliteit in 111 ACS patiënten. Er is met name gekeken naar patroonverschillen tussen patiënten met (cases) en zonder (controls) een herhaaldelijk event (een samengesteld eindpunt bestaande uit cardiovasculaire mortaliteit, myocardinfarct of instabiele angina pectoris waarvoor een urgente coronaire revascularisatie benodigd was) gedurende de follow-up. Uit de resultaten bleek dat post-ACS patiënten met een herhaaldelijk event stabiele en systematisch hogere GDF-15 waarden hadden gedurende de 1-jaars follow-up dan klinisch vergelijkbare post-ACS patiënten zonder een herhaaldelijk event. Daarnaast kwam naar voren dat GDF-15 een lage intra-individuele variabiliteit had. Al met al kan hieruit geconcludeerd worden dat het herhaaldelijk afnemen van GDF-15 tot 1 jaar na het doormaken van een ACS nut heeft om prognosebepalingen te verbeteren, waarbij - in het kader van lage intra-individuele variabiliteit – het aantal herhaalde metingen beperkt kan blijven.

In **Hoofdstuk 9** worden interetnische verschillen bestudeerd van reeds bestaande cardiovasculaire biomarkers in het beloop over de tijd (high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceriden, totaal cholesterol, Troponine T (TnT), NT-proBNP en CRP) in post-ACS patiënten met diabetes mellitus. Tot nu toe zijn cardiovasculaire

biomarkers veelal alleen gevalideerd in Kaukasische populaties en is er weinig bekend over de generaliseerbaarheid van deze biomarkers in andere etnische groepen. Met name in Azië, waar coronaire hartziekten nu opkomend zijn, is er behoefte aan biomarkervalidatie. Derhalve is het beloop over de tijd 1 jaar na een primair ACS event weergegeven voor elke biomarker en vergeleken tussen 48 Kaukasische (Nederlandse) en 48 klinisch vergelijkbare Chinese patiënten. Deze observatie onthulde dat Kaukasische patiënten persisterend hogere LDL, totaal cholesterol, triglyceriden, TnT en CRP waarden hadden dan Chinese patiënten. Er werden geen verschillen gezien in HDL en NT-proBNP waarden tussen de twee cohorten. Deze explorerende analyse zou meer inzicht kunnen geven in bloedbiomarker-gerelateerde verschillen tussen etnische groepen en benadrukt het feit dat bevindingen gebaseerd op Westerse groepen mogelijk niet direct toepasselijk zijn op Chinese individuen.

Hoofdstuk 10 onderzoekt in 135 post-ACS patiënten het tijdsbeloop op de korte (< 30 dagen) en lange (30 dagen - 1 jaar) termijn van eiwit bloedbiomarkers, die de 'intracellular mitogen-activated protein kinase' (MAPK) cascade stimuleren. Patroonverschillen over de tijd tussen patiënten met (cases) en zonder (controls) een herhaaldelijk event (een samengesteld eindpunt bestaande uit cardiovasculaire mortaliteit, myocardinfarct of instabiele angina pectoris waarvoor een urgente coronaire revascularisatie benodigd was) werden met name onderzocht. 'Angiopoietin-1' (ANG-1), 'protease-activated receptor-1' (PAR-1) en 'bone morphogenetic protein-6' (BMP-6) serumwaarden waren significant hoger in patiënten met een herhaaldelijk event in vergelijking met gelijkwaardige patiënten zonder doorgemaakt herhaaldelijk event (controls), maar alleen kort (in de eerste 30 dagen) na een ACS. Derhalve dienen de prognostische eigenschappen van deze signaaleiwitten - die als nieuwe biomarkers dienst zouden kunnen doen - verder onderzocht te worden in de acute fase van een ACS.
Concluderend, de zoektocht naar nieuwe bloedbiomarkers, die relevante pathofysiologische processen in coronairlijden reflecteren, kan leiden tot het identificeren van markers die van nut zouden kunnen zijn voor risicopredictie en zelfs zouden kunnen fungeren als therapeutisch doelwit. Met het oog daarop heeft ons onderzoek geleid tot veelbelovende resultaten met betrekking tot GDF-15, ANG-1, PAR-1 en BMP-6 door het in detail bestuderen van diens patronen over de tijd. Bovendien zijn er relevante verschillen naar boven gekomen tussen Kaukasische en Chinese patiënten in tijdspatronen van reeds bestaande cardiovasculaire biomarkers, wat impliceert dat een etniciteitsspecifieke benadering nodig is.

Het biomarkerbeloop over de tijd is vergeleken tussen cases en controls volgens een 'case-cohort' opzet binnen de *BIOMArCS* studie. Omwille van efficiëntie zijn uiteindelijk

alle cases en een beperkt aantal controls gekozen voor verdere analyse. Deze opzet maakt mogelijk dat klinisch relevante verschillen ontdekt kunnen worden met voldoende statistische power op een efficiënte wijze. De kracht van deze studie zit in het feit dat seriële metingen direct na een ACS (index event) en vóór een ACS (herhaaldelijk event) zijn verricht, wat maakt dat accurate veranderingen in biomarkerpatronen vóór de toetreding van een event geobserveerd kunnen worden. Dit is in tegenstelling tot eerdere studies, die vaak in de vroege, acute fase van een ischemisch event een éénmalige meting hebben verricht. Bovendien reflecteert een verzameling aan metingen beter de stabiliteit van de biomarker gedurende een langere periode dan een éénmalige meting, die onderworpen kan zijn aan incidentele factoren (bijvoorbeeld stress, voeding).

Het is relevant om ook het observationele karakter van de *BIOMArCS* studies te benadrukken, omdat dit impliceert dat causale verbanden niet direct aangetoond kunnen worden. Het moet dus nog uitgezocht worden of de bestudeerde bloedbiomarkers slechts de ziekteprocessen in coronairlijden reflecteren of dat ze daadwerkelijk als 'mediators' bijdragen aan de coronaire pathofysiologie. Ook moet opgemerkt worden dat het ontbrak aan informatie over genetische factoren, omgevingsinvloeden (zoals voeding) en specifieke klinische gegevens over de geïncludeerde patiënten (zoals infarctgrootte of resterende ejectiefractie), die mogelijk circulerende bloedbiomarkers hebben beïnvloed. Deze potentiële verstorende factoren (oftewel 'confounders') werden zoveel als mogelijk vermeden door het matchen van patiënten (cases en controls) op klinische factoren en diagnose bij opname. Tot slot, door het specifiek bestuderen van post-ACS patiënten zoals in het *BIOMArCS* onderzoek, kan niet worden achterhaald of deze bevindingen doorgetrokken kunnen worden naar patiënten met een andere coronaire hartziekte of toegepast kunnen worden op de algemene populatie met betrekking tot primaire preventie.

3. Een betere risicostratificatie en groter behandelvoordeel bij verworven coronaire hartziekten door de bepaling van genetische polymorfismen.

In het derde deel van dit proefschrift is de relatie tussen genetische polymorfismen en cardiovasculaire uitkomsten bestudeerd om de bruikbaarheid ervan te onderzoeken in cardiovasculaire risicostratificatie. Daarnaast is van een aantal genetische varianten bepaald of ze van toegevoegde waarde kunnen zijn in risicoreductie door specifieker ACE-inhibitors te richten op patiënten, die het meeste profiteren van deze middelen. Deze associaties zijn onderzocht in de *PERindopril GENEtic Associations (PERGENE)* studie, een onderdeel van

de *EURopean trial On reduction of cardiac events with Perindopril in stable coronary Artery disease (EUROPA)* studie. De *EUROPA* studie was een gerandomiseerde, dubbelblinde, placebogecontroleerde klinische studie, die opgezet is om het effect van de ACE-inhibitor perindopril te bepalen op cardiovasculaire uitkomsten in 12218 patiënten met stabiel coronairlijden. De cardiovasculaire farmacogenetische *PERGENE* studie was daarvan een substudie, die polymorfismen onderzocht met een mogelijke invloed op het behandelvoordeel van de ACE-inhibitor perindopril op cardiovasculaire uitkomsten. Het laatste hoofdstuk (hoofdstuk 13) van dit deel beschrijft de associatie tussen genetische polymorfismen en coronaire plaque karakteristieken, als onderdeel van de eerder beschreven *ATHEROREMO-IVUS* studie.

Hoofdstuk 11 bevat een omvangrijke analyse van zeven functionele varianten in de 'vascular endothelial growth factor' (VEGF) en 'kinase insert domain-containing receptor' (KDR) genen in relatie tot zowel cardiovasculaire uitkomsten als behandelvoordeel door de ACE-inhibitor perindopril. Er werden uiteindelijk geen associaties gevonden tussen VEGF/KDR polymorfismen of haplotypen met 4-jaars cardiovasculaire uitkomsten in 8711 onderzochte patiënten met stabiel coronairlijden. Daartegenover blijken wel meerdere VEGF/KDR polymorfismen - met name wanneer gecombineerd in de vorm van een farmacogenetische score - het behandeleffect van perindopril op cardiovasculaire uitkomsten te modificeren. Kortom, deze bevindingen ondersteunen dat VEGF/KDR polymorfismen eerder gebruikt zouden kunnen worden om het behandelvoordeel van de ACE-inhibitor perindopril te vergroten met het oog op het reduceren van ongunstige cardiovasculaire uitkomsten, dan dat VEGF/KDR polymorfismen gebruikt zouden kunnen worden in de risicobepaling van stabiel coronairlijden. Een mogelijke interactie tussen VEGF/KDR varianten en ACE-inhibitor behandeling is een nieuwe bevinding, aangezien dit nog niet eerder onderzocht is in klinische studies.

Hoofdstuk 12 focust op twee 'α-adducin' (ADD1) polymorfismen (rs4961 en rs4962) in relatie tot cardiovasculaire uitkomsten, behandeleffect van de ACE-inhibitor perindopril en hypertensie. In een totaal aantal van 9327 patiënten met stabiel coronairlijden, waren de ADD1 polymorfismen niet geassocieerd met cardiovasculaire uitkomsten op een termijn van 4 jaar. Tevens was er geen associatie tussen en de polymorfismen en het effect van perindopril op de bloeddruk of cardiovasculaire uitkomst. Wat betreft hypertensie, hebben alleen dragers van het recessieve allel van de rs4963 variant een significant lagere prevalentie van hypertensie dan hun dominante homozygote equivalenten. Op basis van deze bevindingen kan gesteld

worden dat ADD1 polymorfismen voor zowel toekomstige cardiovasculaire risicopredictie als geïndividualiseerde ACE-inhibitor therapie in patiënten met stabiel coronairlijden geen kans maken.

In **Hoofdstuk 13** is een veelvoorkomende 'haptoglobine' (Hp) polymorfisme onderzocht door middel van de bepaling van eiwitfenotypen (Hp 1-1, Hp 2-1, Hp 2-2) in 585 patiënten, die een coronairangiografie moesten ondergaan omwille van SAP of ACS. De omvang en samenstelling van de coronair atherosclerose is in deze patiënten bepaald met VH-IVUS en 'near-infrared spectroscopy' (NIRS), waarna de relatie werd onderzocht tussen de verschillende Hp fenotypen en deze invasief gemeten coronaire plaque karakteristieken. Er werd een relatie gevonden tussen de Hp 2 fenotypen (Hp 2-1 en Hp 2-2) en de omvang van atherosclerose in ACS patiënten, wat betekent dat ACS patiënten met een Hp 2 fenotype een grotere kans hebben op het ontwikkelen van meer progressieve coronair atherosclerose dan ACS patiënten met een Hp 1 fenotype. Oftewel, genetische verschillen in de endogene anti-oxidante status als weerspiegeling van het Hp fenotype, kan van belang zijn in de risicostratificatie van patiënten die lijden aan coronaire hartziekte.

Globaal genomen zijn er twee hoofdzaken aangestipt in dit deel van het proefschrift waarin specifieke genetische varianten binnen de coronaire hartziekten zijn onderzocht. Allereerst zijn de gespecificeerde polymorfismen op hun potentiële waarde onderzocht als cardiovasculaire risicovoorspellers. Daarin zijn VEGF/KDR en ADD1 polymorfismen niet veelbelovend uit de verf gekomen, maar lijken 'haptoglobine' polymorfismen wel bruikbaar binnen de coronaire risicostratificatie. Daarnaast is bekeken wat de rol is van de onderzochte polymorfismen in het optimaliseren van het behandelvoordeel van ACE-inhibitors bij mensen met stabiel coronairlijden. In dat opzicht is een interactie gevonden tussen VEGF/KDR polymorfismen en het behandeleffect van de ACE-inhibitor perindopril op cardiovasculaire uitkomsten. Deze interactie werd niet gevonden met de onderzochte ADD1 polymorfismen. Deze opgedane kennis kan een stukje informatie bijdragen aan de ontwikkeling van het meer gericht (farmacologisch) behandelen op individueel niveau.

Gesteld moet worden dat de VEGF/KDR en ADD1 polymorfismen zijn geanalyseerd binnen een goed doordachte placebogecontroleerde dubbelblinde gerandomiseerde studie met een grote steekproefomvang en relatief lange follow-up. Echter, sommige aspecten verdienen nadere aandacht. Het ontbreken van een verband tussen de afzonderlijk geanalyseerde polymorfismen en 4-jaars cardiovasculaire uitkomst zou kunnen worden verklaard door het feit dat waarschijnlijk de bijdrage aan prognose van een enkele 'single-nucleotide

polymorphism' (SNP) minimaal is; een individuele SNP kan wellicht geen prognostische informatie alleen dragen. Om die reden zijn specifieke combinaties van een aantal SNPs gemaakt en gevormd tot 'haplotypes' om meer power te verkrijgen. Desondanks bleef een significante associatie tussen de haplotypes en cardiovasculaire uitkomsten uit. Ook moet worden opgemerkt dat de generaliseerbaarheid van de resultaten beperkt is als het gaat om het type patiënt en type geneesmiddel; de gevonden resultaten kunnen naar waarschijnlijkheid niet direct worden doorgetrokken naar alle patiënten met coronaire hartziekten en naar alle soorten ACE-inhibitors. De *PERGENE* studiepopulatie bestond voornamelijk uit mannelijke Kaukasische patiënten met stabiel coronairlijden onder perindopril. Dit betekent dat voorzichtigheid moet worden geboden met de interpretatie van de resultaten in vrouwen, andere bevolkingsgroepen, andere coronaire hartziekten en diverse ACE-inhibitors.

Er kunnen een aantal lessen worden getrokken uit dit proefschrift en als volgt worden samengevat:

Deel 1:

- Herhaaldelijke ongunstige cardiovasculaire events komen nog altijd vaak voor in patiënten met verworven coronairlijden, wat vereist dat kennis op dit gebied verder ontwikkeld moet worden, risicostratificatie / voorspelmethoden verbeterd moet(en) worden en behandelstrategieën verfijnd moeten worden.
- Door externe factoren en circulerende bloedbiomarkers te linken aan invasief gemeten coronaire plaque karakteristieken, kunnen aanvullende pathofysiologische inzichten worden verkregen.
- Zowel roken als circulerend fibrinogeen dragen eerder bij aan de omvang / grootte van coronair atherosclerose dan aan de kwetsbaarheid van de coronaire atherosclerotische plaque, met name in patiënten gediagnosticeerd met ACS.
- Daarentegen heeft circulerend adiponectine laten zien mogelijk wel invloed te hebben op coronaire plaque 'vulnerability' (kwetsbaarheid), met name in patiënten gediagnosticeerd met SAP.
- Verscheidene chemokines (MCP-1, MIP-1α, RANTES) en cytokines (TNF-α, IL-10) zijn geassocieerd met diverse VH-IVUS parameters, die de omvang, compositie en kwetsbaarheid van coronaire atherosclerose beschrijven.
- Hogere waarden van circulerende renale biomarkers (cystatine C en NGAL) zijn

geassocieerd met een lager voorkomen van hoog-risico coronaire laesies in patiënten met coronairlijden, maar niet in diegenen met een slechte nierfunctie.

- Adiponectine, RANTES en cystatine C zijn voorspellend voor ongunstige klinische events gedurende een follow-up van 1 jaar in patiënten met verworven coronairlijden. Dit zijn dus potentiële circulerende biomarkers, die verder onderzocht moeten worden in predictiemodellen om hun toegevoegde waarde vast te stellen in het voorspellen van herhaaldelijke events in coronaire hartziekten.
- Het stratificeren van patiënten op soort coronairlijden (ACS versus SAP) is essentieel om rekening te houden met de verschillen in het onderliggende pathofysiologische substraat.

Deel 2:

- Het zoeken naar nieuwe bloedbiomarkers, die relevante pathofysiologische processen in coronairlijden reflecteren, kan leiden tot het identificeren van markers die bruikbaar kunnen zijn ten behoeve van risicopredictie en zelfs zouden kunnen fungeren als therapeutisch doelwit.
- Eerdere studies op het gebied van bloedbiomarkers in coronairlijden focussen voornamelijk op éénmalige metingen in de vroege, acute fase van een ischemisch event.
- Het bestuderen van het tijdsbeloop van bloedbiomarkers na een ACS event door middel van zeer frequente bloedafnames kan op 4 manieren nuttige informatie opleveren:
  - Het gedrag en de stabiliteit van de marker waarnemen in de acute fase en op lange termijn na een ischemisch event.
  - Het gedragspatroon observeren vóór het optreden van een herhaaldelijk event.
  - De vergelijking maken in biomarkerpatronen tussen patiënten met en zonder het optreden van een herhaaldelijk event.
  - Het bepalen van de intra- en inter-individuele variabiliteit van de marker.
- Post-ACS patiënten die een herhaaldelijk event doormaken hebben stabiele en systematisch verhoogde GDF-15 waarden tijdens 1-jaars follow-up in vergelijking met hun gelijkwaardige controls. Aldus kan herhaald afnemen van GDF-15 gedurende 1 jaar na een ACS de prognosebepaling verbeteren met een beperkt aantal benodigde herhaalde metingen.
- Anderzijds zijn de bloedbiomarkers, die de 'intracellular mitogen-activated protein kinase' (MAPK) cascade stimuleren (ANG-1, PAR-1, BMP-6), alleen voorspellend in

de acute fase voor post-ACS patiënten die een herhaaldelijk event doormaken.

- Er bestaan relevante en standvastige verschillen in tijdspatronen op de lange termijn van reeds bestaande cardiovasculaire biomarkers (LDL, totaal cholesterol, triglycerides, troponin T en CRP) tussen Kaukasische en Chinese post-ACS patiënten, wat de suggestie wekt dat een etniciteitsspecifieke benadering nodig is.

Deel 3:

- Het bestuderen van de relatie tussen genetische polymorfismen en cardiovasculaire uitkomsten is zinvol om het nut van genetica in secundaire cardiovasculaire risicostratificatie te bepalen.
- Varianten in de VEGF, KDR en ADD1 genen zijn niet prognostisch voor ongunstige cardiovasculaire uitkomsten gedurende 4 jaar in patiënten met stabiel coronairlijden.
- Genetische verschillen in de endogene anti-oxidante status als weerspiegeling van het haptoglobine fenotype, kan van belang zijn in de bepaling van het dragen van een hoger risico op uitgebreidere coronaire atherosclerose in ACS patiënten.
- Genetische varianten kunnen worden gebruikt om farmacologische behandeling specifieker te richten op patiënten, die daar het meest baat van zouden kunnen hebben en om op die manier bij te dragen aan ‘personalized medicine’.
- Verscheidene VEGF/KDR polymorfismen bleken van invloed te zijn op het behandelresultaat van perindopril op ongustige cardiovasculaire uitkomsten in patiënten met stabiel coronairlijden.

List of publications

Buljubasic N, Akkerhuis KM, de Boer SP, Cheng JM, Garcia-Garcia HM, Lenzen MJ, Oemrawsingh RM, Battes LC, Rijndertse M, Regar E, Serruys PW, van Geuns RJ, Boersma E, Kardys I. Smoking in relation to coronary atherosclerotic plaque burden, volume and composition on intravascular ultrasound. *PLoS One 2015; 10(10): e0141093. doi: 10.1371/journal.pone.0141093.*

Buljubasic N, Akkerhuis KM, Cheng JM, Oemrawsingh RM, Garcia-Garcia HM, de Boer SP, Regar E, van Geuns RM, Serruys PW, Boersma E, Kardys I. Fibrinogen in relation to degree and composition of coronary plaque on intravascular ultrasound in patients undergoing coronary angiography. *Coron Artery Dis 2017; 28(1): 23-32. doi: 10.1097/MCA.0000000000000442.*

Marino BCA, **Buljubasic N**, Akkerhuis M, Cheng JM, Garcia-Garcia HM, Regar E, Geuns RV, Serruys PW, Boersma E, Kardys I. Adiponectin in relation to coronary plaque characteristics on radiofrequency Intravascular ultrasound and cardiovascular outcome. *Arq Bras Cardiol 2018; 111(3): 345-353. doi: 10.5935/abc.20180172.*

Cheng JM, Oemrawsingh RM, Akkerhuis KM, Garcia-Garcia HM, de Boer SP, Battes LC, **Buljubasic N**, Lenzen MJ, de Jaegere PP, van Geuns RJ, Serruys PW, Kardys I, Boersma E. Circulating chemokines in relation to coronary plaque characteristics on radiofrequency intravascular ultrasound and cardiovascular outcome. *Biomarkers 2014; 19(7): 611-619. doi: 10.3109/1354750X.2014.957725.*

Battes LC, Cheng JM, Oemrawsingh RM, Boersma E, Garcia-Garcia HM, de Boer SP, **Buljubasic N**, Mieghem NA, Regar E, Geuns RJ, Serruys PW, Akkerhuis KM, Kardys I. Circulating cytokines in relation to the extent and composition of coronary atherosclerosis: Results from the ATHEROREMO-IVUS study. *Atherosclerosis 2014; 236(1): 18-24. doi: 10.1016/j.atherosclerosis.2014.06.010.*

Brankovic M, Akkerhuis KM, **Buljubasic N**, Cheng JM, Oemrawsingh RM, Garcia-Garcia HM, Regar E, Serruys PW, van Geuns RJ, Boersma E, Kardys I. Plasma Cystatin C and Neutrophil Gelatinase Associated Lipocalin in relation to coronary atherosclerosis on intravascular ultrasound and cardiovascular outcome: Impact of kidney function (ATHEROREMO-IVUS study). *Atherosclerosis 2016; 254: 20-27. doi: 10.1016/j.atherosclerosis.2016.09.016.*

Buljubasic N, Vroegindewey MM, Oemrawsingh RM, Asselbergs FW, Cramer E, Liem A, van der Harst P, Maas A, Ronner E, Schotborgh C, Wardeh AJ, Akkerhuis KM, Boersma E. Temporal pattern of Growth Differentiation Factor-15 (GDF-15) protein after acute coronary syndrome (from the BIOMArCS study). *Am J Cardiol 2019; 124(1): 8-13. doi: 10.1016/j.amjcard.2019.03.049.*

Buljubasic N, Zhao W, Cheng JM, Li H, Oemrawsingh RM, Akkerhuis KM, Yu H, Zhou L, Wu Y, Boersma E, Gao W. Comparison of temporal changes in established cardiovascular biomarkers after acute coronary syndrome admission between Caucasian and Chinese patients with diabetes mellitus. *Submitted.*

Vroegindewey MM, **Buljubasic N**, Oemrawsingh RM, Kardys I, Asselbergs FW, van der Harst P, Umans VA, Kietselaer B, Lenderink T, Liem A, Mouthaan H, Boersma E, Akkerhuis KM. Serum biomarkers that stimulate the Mitogen-Activated Protein Kinase cascade in relation to recurrent coronary events following an acute coronary syndrome. *J Mol Biomark Diagn 2019; 10(2): 414. doi: 10.4172/2155-9929.1000414.*

Buljubasic N, Kappers MHW, Dehghan A, Brugts JJ, Versmissen J, Remme WJ, Bertrand ME, Fox KM, Ferrari R, Simoons ML, Boersma E, Kardys I, Akkerhuis KM. Vascular Endothelial Growth Factor (-Receptor) polymorphisms in relation to cardiovascular outcome and response to ACE-inhibitor therapy: An analysis from the PERindopril GENEtic association study. *Submitted.*

Oemrawsingh RM, **Buljubasic N**, Brugts JJ, Van Vark LC, Remme WJ, Bertrand ME, Fox KM, Ferrari R, Danser AHJ, De Maat M, Simoons ML, Akkerhuis KM, Boersma E. α-Adducin gene variants in hypertension and response to ACE-inhibitor therapy: results of the PERindopril GENEtic association study. *Submitted.*

Buljubasic N, Oemrawsingh RM, Smeets MB, Cheng JM, Regar E, van Geuns RJ, Serruys PW, Boersma E, Akkerhuis KM, Kardys I, Arslan F. Haptoglobin polymorphism in relation to coronary plaque characteristics on radiofrequency intravascular ultrasound and near-infrared spectroscopy in patients with coronary artery disease. *Int J Cardiol 2016; 15; 221: 682-687. doi: 10.1016/j.ijcard.2016.07.126.*

About the author

Nermina Buljubašić was born on February 22nd, 1990 in Loznica (the former republic of Yugoslavia). As soon as the Yugoslav war broke out, she fled with her parents and sister to the Netherlands in 1992.

After finishing secondary education (Gymnasium) cum laude in the Netherlands, she started studying Medicine at the Erasmus Medical Center, Rotterdam (NL) in 2008. During her second year of Medicine, she got invited by the Dean of the university to participate in an extracurricular research training programme, based on her grades. Subsequently, she started a Master of Science programme in Clinical Research at the Netherlands Institute for Health Sciences (NIHES) in 2010, in parallel to Medicine. As part of the programme, she completed several research courses at the Harvard School of Public Health in Boston (USA) and the University of Cambridge (UK). She successfully finished the Clinical Research training in 2014 and obtained a Master of Science degree, whereafter she graduated in Medicine in 2015.

After obtaining her medical degree, she started working as a PhD candidate in the research group of prof. dr. Eric Boersma at the department of Cardiology, Erasmus Medical Center, Rotterdam (NL) in the end of 2015. She worked on several research projects concerning blood biomarkers in coronary artery disease.

When she entered the final stage of her PhD trajectory at the end of 2017, she started to work at the department of Internal Medicine in Amphia Ziekenhuis, Breda (NL) and afterwards within the field of Geriatric Medicine / Elderly Care in several nursing and rehabilitation homes of Rotterdam (NL) to broaden her vision and obtain clinical experience.

In August of 2019, love made her move to Southern Denmark (Kolding). In September of 2019, she started a high-speed medical Danish language course and after 3 months a clinical internship at hospital 'Lillebælt' (Sygehus Lillebælt) in order to further pursue a medical career in Denmark.

PhD portfolio

Summary of PhD training and teaching activities

1. PhD training	Year	Workload (ECTS)
Research skills		
- 'BROK' course on scientific integrity	2017	1.5
- Master of Science in Clinical Research (NIHES)	2010 - 2014	70.0
General academic skills		
- Biomedical English Writing course	2012	3.0
In-depth courses		
- COEUR 'Atherosclerotic and aneurysmal disease'	2012	1.5
- COEUR 'Vascular clinical epidemiology'	2012	1.5
- COEUR 'Cardiovascular pharmacology'	2013	1.5
- COEUR 'Pathophysiology of ischemic heart disease'	2014	1.5
- COEUR 'Cardiovascular medicine'	2015	1.5
- COEUR 'Cardiovascular imaging and diagnostics'	2015	1.5
- COEUR 'Heart failure research'	2016	1.5
- COEUR 'Atherosclerotic plaque imaging'	2017	1.5
International conferences and symposia		
- EuroPrevent ESC congress 2018, Ljubljana, Slovenia	2018	1.5
Seminars and workshops		
- PhD training course 'Atherosclerosis & Thrombosis', organized by the Dutch Heart Foundation in Arnhem, the Netherlands	2017	6.0

2. Teaching activities	Year	Workload (ECTS)
Lecturing		
- Journal club (research group meeting): 'Repeated measurements'	2012	0.3
- Journal club (research group meeting): 'Non-inferiority trials'	2012	0.3
- Journal club (research group meeting): 'Multiple imputation for missing data'	2013	0.3
- Journal club (research group meeting): 'Crossed or nested random effects in linear mixed models'	2017	0.3
- Journal club (staflunch): 'After Eighty study'	2016	0.3
- Teaching assistant in the 'Basic introduction course on SPSS'	2016 & 2017	0.6
Supervision of students		
- Second year medical students: performing a systematic review	2016 & 2017	0.6
Presentations		
- EuroPrevent ESC congress 2018, Ljubljana, Slovenia (poster & oral presentation)	2018	0.3
Total		97

Dankwoord / Acknowledgements

Graag wil ik alle patiënten, onderzoekers, begeleiders, collega's, vrienden & familie bedanken voor hun betrokkenheid aan de totstandkoming van dit proefschrift. Hierbij wil ik - in 4 verschillende talen - een aantal mensen in het bijzonder bedanken.

Allereerst mijn promotor, prof. dr. ir. H. Boersma. Beste **Eric**, onze eerste kennismaking dateert alweer van een geruime tijd terug tijdens mijn eerste lessen over statistiek. Als geneeskunde studente, die net was begonnen aan een research master en nog helemaal niets begreep van onderzoek doen, zat ik altijd vooraan de rij bij jou in de klas. Iemand die zo'n moeilijk onderwerp op zo'n eenvoudige, geduldige, didactische wijze kon overbrengen en het zelfs nog leuk maakte ook, moest in mijn ogen een goede begeleider zijn om onderzoek bij te doen. En zo geschiedde. Nadat ik mijn interesse voor onderzoek binnen de cardiologie kenbaar had gemaakt en onderzoekservaring wilde opdoen in jouw onderzoeksgroep als studente, kon ik meteen aan de slag en aansluiten bij een aantal lopende onderzoeksprojecten. Je bracht me op het rechte pad en bood mij de kans om direct een promotietraject in te rollen na de studie geneeskunde. Veel dank voor deze mogelijkheid! Ik zal niet alleen met veel plezier aan jou terugdenken als onderzoeks(bege)leider, die altijd (laagdrempelig) klaarstaat voor zijn PhD studenten, maar ook als mens: bescheiden, oprecht en loyaal.

Mijn copromotoren, Dr. I. Kardys en Dr. K.M. Akkerhuis, ben ik uiteraard ook veel dank verschuldigd. **Isabella**, jij stond altijd klaar om te sparren over literatuur, te helpen met statistische analyses, resultaten inzichtelijk te maken en de kunst over te brengen van het schrijven. Ik heb veel van je geleerd! Daarnaast wil ik je bedanken voor alle bemoedigende woorden en de momenten waarop je me bijstuurde, als ik weer teveel was afgedwaald in statistische details, waar ik mijn vingers maar beter niet aan kon branden. **Martijn**, dank voor je oprechte interesse, adviezen en betrokkenheid vanaf het begin tot het einde. Je hebt een steentje bijgedragen aan dit proefschrift door het klinisch, wetenschappelijk en taalkundig naar een hoger niveau te tillen. Je hebt de nodige artikelen altijd met veel aandacht en precisie bekeken en van nuttig inhoudelijk commentaar voorzien. Ook ben je altijd betrokken geweest in de keuzes die ik gaandeweg heb gemaakt, waarvoor veel dank.

De promotiecommissie wil ik bedanken voor hun bereidwilligheid en tijd om mijn proefschrift te beoordelen: **prof. dr. R.H.N. van Schaik** (Erasmus Medisch Centrum, Rotterdam), **prof. dr. R.J. de Winter** (Academisch Medisch Centrum Amsterdam), **prof. dr. F.W. Asselbergs** (Universitair Medisch Centrum Utrecht), **prof. dr. F. Zijlstra** (Erasmus Medisch Centrum, Rotterdam), **dr. J.E. Roeters van Lennep** (Erasmus Medisch Centrum, Rotterdam).

Mijn dank gaat ook uit naar alle co-auteurs, zonder wie dit proefschrift nooit tot stand gekomen zou zijn. In het bijzonder **Sanneke**, die mij als studente heeft begeleid en zich in heeft gezet voor het schrijven van mijn eerste artikel. De eerste stap was daarmee gezet. **Jin**, **Laura** en **Linda**, bedankt voor jullie gezelligheid, betrokkenheid en inspirerende enthousiasme, toen ik als studente bij jullie op de kamer / in de kelder zat! **Rohit**, dank voor de leuke samenwerking en de scherpe blik op alle artikelen – van AtheroRemo tot PERGENE tot BIOMArCS. **Mariëtte**, dank voor het 'VEGF / KDR polymorfismen' idee en de fijne, laagdrempelige supervisie op de interne geneeskunde afdeling van het Amphia Ziekenhuis in Breda.

Veel dank natuurlijk ook aan **alle collega's** van de **Klinische Epidemiologie (KLEP) groep**, vanaf het trialbureau tot aan mijn kamergenoten, voor een prettige sfeer en fijne samenwerking. **Maxime**, hoe ééntonig zou het dagelijkse onderzoeksleven in Ba-559, Bd-414 en Z-520 zijn geweest zonder jou. Bedankt voor je flinke dosis humor, het dolce gusto koffieapparaat in de kamer, de afgespeelde spotify lijsten en alle gezellige gesprekken. **Miloš**: veni, vidi, vici. You came, you saw, and you conquered. It has been a pleasure to work together with such an intelligent, talented, passionate and dedicated person. Thanks for the many talks and laughs we shared in the office, at lunch and social events. I hope we can go for a 'bakkie pleur' some day again in Rotterdam! **Victor**: 'niet lullen, maar poetsen'. Dank voor je goede dosis humor en gezonde dosis nuchterheid op de momenten dat ik me om de kleinste dingen druk kon maken. Ik wens je alle goeds toe met **Cordula** en jullie kinderen. **Sharda**, dankjewel voor de rust, vriendelijkheid en warmte die je altijd uitstraalde. Het was een fijne tijd met jou op kantoor. **Anne-Sophie**, ondanks dat we nooit een kantoor deelden, voelde het toch alsof je al die tijd een kamergenoot was. De dagelijkse kletssessies, gezellige 'loopjes' naar de AH to go en ons avontuur in Papendal zal ik niet snel vergeten. **Elke**, ook jij was een onmisbare kamergenoot 'op afstand' en een rots in de branding voor de groep. Dank voor altijd een luisterend oor, je behulpzaamheid en positiviteit. **Jan-Kees**, ik zou jou zo

weer kiezen om mee samen te werken als begeleider voor studenten van systematic reviews. Bedankt voor alle leuke tijden en goede gesprekken. **Sara**, dankjewel dat ik altijd bij je aan mocht kloppen voor statistische vraagstukken. **Michelle**, **Marie**, **Nathalie** en **Muhammed**, jammer dat ik jullie allen pas in het eindtraject heb leren kennen - bedankt voor de gezellige laatste maanden in het Ba-gebouw op de 5e verdieping en in de kantoortuin. **Colinda**, een enthousiaste toevoeging aan de KLEP groep halverwege mijn promotietraject. Veel dank voor de leuke samenwerking rondom het BioSHiFT project en onze uitgebreide lunchsessies in Doppio. Je betrokkenheid, behulpzaamheid en interesse heb ik altijd zeer gewaardeerd. **Yvonne**, bedankt voor de organisatorische hulp achter de schermen.

Gedurende het promotietraject, heb ik ook klinische ervaring opgedaan. Dank aan alle collega's en opleider **Joost** (**Dr. van Esser**) van de interne geneeskunde in het Amphia Ziekenhuis, waar ik een onvergetelijke tijd heb gehad. Dank ook aan het fantastische team van **GeriCall**, waar ik veel van geleerd heb en de nodige tijd kreeg om de laatste loodjes van het proefschrift af te maken.

Wat moet een meisje zonder haar (beste) vriendinnen? Ook jullie wil ik stuk voor stuk bedanken, omdat jullie van grote betekenis zijn geweest voor de nodige ontspanning. Van alle mensen, die beschreven staan in het dankwoord, ken ik **Laressa** toch wel het langste. Van kleins af aan konden we het al goed met elkaar vinden. Van stoepkrijten, skelter rijden, belletje trekken tot aan weekendjes weg, musicals, theater, high tea en lunchdates. Dankjewel dat je al die jaren zoveel interesse hebt gehad in wat ik deed en wat mij allemaal bezighield. Je bent een fantastische meid en een betrouwbare vriendin. Laten we deze bijzondere vriendschap nog vele jaren voortzetten - ondanks de afstand. **Cindy** & **Willeke**, jullie horen daar natuurlijk ook bij. Bedankt voor alle supergezellige momenten samen. Ik kan ontzettend om en met jullie lachen! Ik ga het missen nu we niet meer zo vaak kunnen afspreken. **Minka**, we begonnen als 'ambassador' collega's voor de EUR, eindigden als vriendinnen en later zelfs als buren. De deur zal altijd voor je openstaan, ook in Denemarken. **Stephanie**, wie had ooit aan het begin van de coschappen gedacht dat jij uiteindelijk mijn belangrijkste koffiemaatje in dit hele promotietraject zou zijn? Veel dank aan jou en **Krishna** voor de leuke intermezzo's tussen alle data, analyses en het schrijven door. Ik zal de babyccino's met Faye, het ontbijten, lunchen, koffieleuten en cocktails drinken in de stad heel erg missen. **Mounira**, wat bewonder ik jou als persoon, dokter, echtgenote en moeder. Je bent een mooi mens.

Lieve **Sanaz**, wat bijzonder om jou als goede vriendin en paranimf aan mijn zijde te hebben. Met jou was het een 'klik op het eerste gezicht' toen we elkaar ontmoetten op de eerste dag van de studie geneeskunde. Je begrijpt mij als geen ander, we lachen om dezelfde stomme dingen en raken nooit uitgepraat. Mijn bewondering is groot voor de stappen die je aan het zetten bent binnen de plastische chirurgie. Ik zou het je niet na kunnen doen.

Liefste **Jasmina**, mijn grote zus, vriendin voor het leven en natuurlijk ook paranimf. Wij kennen elkaar als geen ander: we zijn zo verschillend, maar tegelijkertijd ook zo hetzelfde. Ontzettend bedankt voor alle onvoorwaardelijke steun en zorgzaamheid. Je bent een goudeerlijke, onafhankelijke en ijzersterke vrouw. Weet dat je ook altijd op mij kunt rekenen: 723 kilometer verandert daar niets aan. Wij zullen altijd aan elkaars zijde staan en ik kijk uit naar nog veel meer onvergetelijke avonturen samen!

At the end of this journey, I moved to Denmark, where I started to learn the language with a fantastic group of medical professionals. We started as strangers, but soon ended up like a family. Thank you all so much for your interest and compassion for my PhD thesis. Dear **Maria**, teaching Danish is a work of (he)art. Thank you for inspiring and motivating us every day by your unique way of teaching. You have not only shown to be a passionate and excellent teacher, but also a kind person with a beautiful heart. Thank you for your understanding, support and all you have done for me so far to make a great start in Denmark. It's simply amazing how you make it possible to get us start working already after 3 months. And please don't kill me for writing this section in English. **Elise**, what a pleasure it is to have you as a teacher as well. With you we learn, but also laugh a lot – which makes it a joy to come to class. Thank you for your kindness, patience and interest. **Lisa**, Danmarks bedste sygeplejerske fra Italien! Without you, learning Danish would not be the same. Thanks for the daily laughs you bring to our classes, car rides and coffeedates. **Dirk**, who would have thought that having lunch, singing songs and a daily drive with two girls in your car – is not *that* bad? We couldn't have imagined the group without you! **Myriam**, we both know what it means to move to another country for love. Your positivity and look on the future is inspiring. **Cristina**, I recognize myself so much in you. 'Du skal tale perfekt Dansk' – I am sure we will get there one day! **Esther**, wat was ik blij dat er een leuke, spontane, Nederlandse meid aan de groep werd toegevoegd. Laten we er wat van gaan maken in Denemarken!

It's not the place, it's the people that make you feel like home. I want to thank the entire family-in-law for their warm welcome and genuine interest in everything I do. **Samija** & **Muenes**, od prvog dana sam osjetila vašu toplinu, pozitivnu energiju i podršku. Hvala vam na sve što ste učinili za mene da se osjećam kao kod kuće. **Admir** & **Lamija**, I could have not wished for a better brother-in-law and a sweeter sister-in-law. Together with **Tarik** & **Amra**, I want to thank all of you for your incredible kindness. Your understanding, support and interest is deeply appreciated. Looking forward to make many more new memories together. A special thanks to **Semra** & **Melisa** for their warm welcome in Denmark the first week in September and for sharing my love for sushi.

Min kære **Haris**, it's finally done. Never in a million years I could have guessed the turn my life would get when we met 2.5 years ago. Thank you for making me smile every day since then. Thank you for your positivity and inspiring look on life. Thank you for believing in me, even when I didn't. Thank you for learning me to take risks and drop fears. Without your commitment, perseverance, tranquility, understanding and care, we would have not come this far. I look back to wonderful memories along this journey and let's enjoy all that is ahead of us. Together we move mountains!

Tot slot bedank ik mijn liefste **ouders**, moji tata i mama. Dit proefschrift is voor jullie en dankzij jullie. In de bloei van jullie leven, zijn jullie afgereisd naar het onbekende met twee kleine kinderen en slechts één koffer in de hand. Jullie hebben huis en haard verlaten om noodgedwongen ergens anders weer opnieuw te beginnen. Mijn dank voor wat jullie opgeofferd hebben om mij en Jasmina een gouden toekomst te geven, is niet in woorden uit te drukken. Dankzij jullie eerlijkheid, wijze adviezen, belangstelling, geborgenheid en bemoediging, zijn wij zo ver gekomen. Ik hoop dat ik minstens net zoveel voor jullie terug zal kunnen doen.